Mandelate Dehydrogenases of *Acinetobacter calcoaceticus*

by

Celia A. Hills

Thesis submitted to the

University of Glasgow

for the degree of

Doctor of Philosophy

Department of Biochemistry

November, 1979

ACKNOWLEDGEMENTS

I wish to thank Professor R.M.S. Smellie and Professor A.R. Williamson for making available the facilities of the Department of Biochemistry, and the Medical Research Council for awarding me a studentship.

My sincere thanks go to Dr C.A. Fewson for his endless advice, guidance and patience.

I would like to acknowledge the excellent assistance received from both Alan Scott, during growth and harvesting of large cultures of bacteria and for doing some of the many phenylglyoxylate carboxy-lyase assays, and Dorothy Brebner for carrying out characterization tests on many of the bacterial strains used in this thesis. I am also very grateful to all my colleagues in laboratory C24 for their critical discussion and suggestions.

Some of the equipment and materials used in this research were purchased with grants to Dr Holms and Dr Fewson from the Royal Society, the Medical Research Council and the Science Research Council.

I am grateful to Mrs. J. Greenwood for her patience and understanding during the typing of this thesis. Finally, I wish to thank John Lavender for his encouragement and invaluable assistance with proof reading.

ABBREVIATIONS

Abbreviations used in this thesis follow the Biochemical Journal Instructions to Authors (revised 1978) with the following additions:

NCIB	National Collection of Industrial Bacteria
ATCC	American Type Culture Collection
D, DMDH	D(-)-mandelate dehydrogenase
L, LMDH	L(+)-mandelate dehydrogenase
P, PC	phenylglyoxylate carboxy-lyase
BDH	benzaldehyde dehydrogenase
BADH	benzyl alcohol dehydrogenase
CO	catechol 1,2-oxygenase
BO	benzoate oxidase
o	no enzyme activity
i	inducible enzyme activity
c	constitutive enzyme activity
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
DCIP	2,6-dichlorophenolindophenol
PMS	N-methyl-phenazonium methosulphate (phenazine methosulphate)
TPP	thiamin pyrophosphate
NEM	N-ethylmaleimide
μ	specific growth rate

CONTENTS

		page
TITLE PAGE		i
ACKNOWLEDGEMENTS		ii
ABBREVIATIONS		iii
CONTENTS		iv
SUMMARY		xiii
INTRODUCTION		
1.	Acinetobacter	1
2.	Metabolism of mandelate and related compounds	4
3.	Regulation of the mandelate pathway	19
4.	Comparison of the evolution and regulation of the β-ketoadipate and mandelate pathways	24
5.	Experimental evolution in bacteria	27
6.	Aims of this work	42
7.	Mutants previously isolated from A. calcoaceticus NCIB8250	45
METHODS		
1.	Materials	48
2.	Microbiological techniques	50
2.1	Microorganisms	50
2.2	Characterization of the bacteria	53
2.3	Storage of the bacteria	53
3.	Sterilization	53
3.1	Moist heat	53
3.2	Dry heat	56
3.3	Filtration	56
3.4	Ethylene oxide	56
4.	Media	57
4.1	Defined media	57
4.2	Nutrient broth	57
4.3	Agar plates	58
4.3.1	Nutrient agar plates	58
4.3.2	Agar plates containing defined media	58

		page
5.	Turbidity measurement	58
6.	Growth of bacteria	59
6.1	Growth of cultures	59
6.2	Nutrient broth inocula	60
6.3	Growth of strains EBF65/65, NCIB8250 and mutants derived from NCIB8250, on the carbon sources included as part of the characterization experiment	60
6.4	Growth of mutants isolated from strain 41, able to grow well on benzyl alcohol, for the measurement of phenylglyoxylate carboxy-lyase activity	60
6.5	Growth of mutants HMM5, 148, 219 and D6 in 5mM-L-glutamate/salts P2 medium to determine the effect of growth temperature on D- and L-mandelate dehydrogenase activity	61
6.6	Growth of mutant strains derived from strains D4OE, 41 and HMM5 in 5mM-L-glutamate/salts P2 medium for the measurement of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase activities	61
6.7	Growth of mutant 148 in 5mM-D-mandelate + 5mM-L-glutamate/salts medium for development of the D-mandelate dehydrogenase assay	61
6.8	Growth of cultures in 5mM-L-glutamate/salts P2 medium with and without additional inducers, repressors or anti-inducer	62
6.9	Growth of mutant HMM5 in 5mM-L-glutamate/salts P2 medium at 30°C	62
6.10	Growth of mutant HMM5 in 40mM-L-glutamate/salts P2 medium	62
6.11	Growth of the wild-type strain EBF65/65, and mutants derived from this strain	63
7.	Harvesting and storage of bacteria	63
7.1	Routine method	63
7.2	Harvesting and storage of strain 41, wild-type strain EBF65/65 and mutants derived from these strains for measurement of phenylglyoxylate carboxy-lyase activity	64
8.	Isolation of mutants	64
8.1	Isolation and identification of mutants constitutive for phenylglyoxylate carboxy-lyase	64
8.2	Isolation of mutants of NCIB8250 and NF1408 able to grow on D- or L-mandelate	65
8.3	Isolation of mutants of strain C48 able to grow on L-mandelate	67

		page
9.	Disruption of bacteria	67
9.1	Preparation of extract	67
9.1.1	Preparation of extract for measurement of D- and L-mandelate dehydrogenase, benzaldehyde dehydrogenase catechol 1,2-oxygenase, and NADH oxidase	67
9.1.2	Preparation of extract of mutant 219 for comparison of D- and L-mandelate dehydrogenases	68
9.2	Preparation of a membrane fraction of mutant 219 to identify the reaction product from the oxidation of D-mandelate and to measure oxygen uptake	68
10.	Treating bacteria with toluene	69
11.	Enzyme assays	69
11.1	D-Mandelate dehydrogenase	70
11.2	L-Mandelate dehydrogenase	71
11.2.1	Original L-mandelate dehydrogenase	71
11.2.2	Modified L-mandelate dehydrogenase	72
11.3	Phenylglyoxylate carboxy-lyase	72
11.4	Benzaldehyde dehydrogenase	73
11.5	Benzyl alcohol dehydrogenase	73
11.6	NADH oxidase	74
11.7	Catechol 1,2-oxygenase	74
12.	Identification of mandelate pathway intermediates	75
12.1	Phenylglyoxylate in the presence of D-mandelate	75
12.2	Benzaldehyde	75
13.	Thin layer chromatography	75
14.	Preparation and chromatography of 2,4-dinitrophenyl-hydrazones	77
15.	Measurements of oxygen uptake	78
15.1	Measurement of 'benzoate oxidase' in the oxygen electrode	79
16.	Charcoal treatment of bovine serum albumin	79
17.	Melting-point determination	79
18.	Optical rotation	80
19.	pH measurement	80
20.	Protein estimation	80
21.	Statistical methods	81
22.	Glassware	81
22.1	General glassware	81
22.2	Pipettes	81
23.	Safety	82

RESULTS

		page
1.	Development of methods for growth of bacteria	83
1.1	Problem of lysis	85
1.2	Temperature of growth	88
1.3	Constituents of growth medium	92
2.	The product of D-mandelate oxidation	93
2.1	Identification of phenylglyoxylate as the reaction product	95
2.2	Quantitative estimation of the phenylglyoxylate produced by D-mandelate oxidation	96
3.	Development and assessment of a method for assaying D-mandelate dehydrogenase in extracts	100
3.1	Ultrasonic disruption of bacteria for the measurement of enzyme activity	100
3.2	Development of an assay procedure for D-mandelate dehydrogenase	101
3.3	Effect of the activity of the extract on the measurement of D-mandelate dehydrogenase	109
3.4	The effect of extract volume on the measured D-mandelate dehydrogenase activity	110
3.5	Variations due to growing bacteria on different days	113
3.6	Reproducibility of the disruption and assay procedures	113
3.6.1	Variations due to ultrasonic disruption	113
3.6.2	Variations amongst assays	113
3.7	Effects of freezing and storing on the activity of D-mandelate dehydrogenase	116
4.	Oxygen uptake	118
5.	Comparison of the properties of D- and L-mandelate dehydrogenase	122
5.1	Determination of K_m and K_i for D- and L-mandelate dehydrogenase	124
5.2	Temperature dependence of the D- and L-mandelate dehydrogenase reactions	126
5.3	Effect of reagents which react with sulphydryl groups on the activities of D- and L-mandelate dehydrogenase	126
5.4	Effect of metal chelating reagents and related compounds on the activities of D- and L-mandelate dehydrogenase	129
5.5	Effect of pH on the activity of D- and L-mandelate dehydrogenase	131

		page
6.	The regulation of D-mandelate dehydrogenase in mutants of A. calcoaceticus NCIB8250	133
6.1	Control of D-mandelate dehydrogenase in strain 41 under conditions of induction, repression and anti-induction	133
6.2	Isolation of constitutive mutants from strains D4OE and 41	137
6.2.1	Frequency of appearance of strains D4OE and 41 becoming constitutive for phenylglyoxylate carboxy-lyase	139
6.2.2	Enzyme activities measured in mutants derived from strains 41, D4OE and HMM5 which constitutively synthesize phenylglyoxylate carboxy-lyase	139
6.2.3	Measurement of the mandelate pathway enzymes in various mutants derived from wild-type strain NCIB8250	144
6.2.4	Phenylglyoxylate carboxy-lyase activity in mutants isolated from strain 41 able to grow well on benzyl alcohol plates	145
7.	Isolation of mutants from A. calcoaceticus EBF65/65, mutant strain C48	150
7.1	Measurement of the enzymes of the mandelate pathway in mutants derived from strains C48 and 61c	154
8.	Comparison of the D- and L-mandelate dehydrogenases from mutants 219 and 364	156
9.	Enrichment of mutants with evolved mandelate dehydrogenases from strains NCIB8250, NF1408 and C48	159
9.1	Estimation of frequency of mutation of strains NCIB8250 and NF1408 to utilize D-mandelate, and strain NF1408 to utilize L-mandelate	159
9.2	Summary of frequency of appearance of mutants from strain NCIB8250, NF1408 and C48 able to utilize D- or L-mandelate	160

DISCUSSION

1.	Lysis of cultures	164
2.	The initial step in the metabolism of D-mandelate	166
2.1	The existence of a specific D-mandelate dehydrogenase	166
2.2	The D-mandelate dehydrogenase assay	168
2.3	Reliability of the D-mandelate dehydrogenase assay	172
3.	Comparison of the mandelate dehydrogenases	175
4.	The regulation of the evolved mandelate dehydrogenases	182
5.	Evolution of the new mandelate dehydrogenase	188
6.	Speculations concerning control and organisation of the genes of the mandelate pathway	197
7.	General conclusions and prospects	199

REFERENCES 200

Scheme

		page
1	The β-ketoadipate pathway for the metabolism of catechol and protocatechuate in bacteria	6
2	Pathway of L-mandelate and benzyl alcohol metabolism in *Acinetobacter calcoaceticus* NCIB8250	8
3	Pathways for mandelate oxidation to benzoate in three microorganisms	11
4	Proposed pathway for phenylalanine degradation in a strain of *Aspergillus niger* isolated from soil	14
5	Proposed pathway for the degradation of phenylacetate by *Aspergillus niger* UBC814	16
6	Metabolism of mandelate in various microorganisms	17
7	Induction of enzymes for the metabolism of mandelate	20
8	Metabolism of xylitol in a mutant of *Klebsiella aerogenes* 1033	30
9	Metabolism of xylitol in a mutant of *Escherichia coli* K12	30
10	Metabolism of D-lyxose in a mutant of *Escherichia coli* K12	34
11	Metabolism of D-arabinose by L-fucose enzymes in a mutant of *Escherichia coli* K12	34
12	Mutants previously isolated from *Acinetobacter calcoaceticus* NCIB8250	46
13	Mutants isolated from *Acinetobacter calcoaceticus* EBF65/65	151
14	A summary of mutants isolated from *Acinetobacter calcoaceticus* NCIB8250	183

Figure

		page
1	Standard curve for the estimation of phenylglyoxylate in the presence of D-mandelate	76
2	An example of lysis whilst growing mutant 219 in 5mM-L-glutamate/salts P2 medium	86
3	Heat inactivation of D- and L-mandelate dehydrogenase in suspensions of intact bacteria	91
4	Absorption spectra of phenylglyoxylate and the reaction product of the oxidation of D-mandelate	94
5	Absorption spectra of 2,4-dinitrophenylhydrazones	97
6	Formation of phenylglyoxylate from D-mandelate by membrane preparations of mutant 219	98
7	The effect of PMS and BSA on the activity of D-mandelate dehydrogenase	105
8	The effects of increasing protein concentration and the omission of BSA and PMS from the reaction mixture to measure D-mandelate dehydrogenase activity	106
9	Effect of specific activity of extract on the measurement of D-mandelate dehydrogenase	111
10	Effect of extract volume on the measured D-mandelate dehydrogenase activity	112
11	Measurement of oxygen uptake with D-mandelate for a membrane fraction of mutant 219	120
12	The effect of PMS on the activity of L-mandelate dehydrogenase	123
13	The effect of BSA on the activity of L-mandelate dehydrogenase	123
14	Determination of K_m and K_i for L-mandelate dehydrogenase	125
15	Temperature dependence of the D- and L-mandelate dehydrogenase reactions	127
16	Effect of pH on the activity of D- and L-mandelate dehydrogenase	132
17	Correlation of induction of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase in mutant 41 under a range of conditions	136
18	Growth of wild-type NCIB8250 and mutants on solid media containing phenylglyoxylate or benzyl alcohol as carbon source	138
19	Growth of mutants of strain EBF65/65 on solid medium containing L-mandelate, D-mandelate, or phenylglyoxylate + 2-phenylpropionate	153
20	Heat inactivation of D- and L-mandelate dehydrogenase of mutants 219 and 364	158

Table

		page
1	First or second-stage mutants of A. calcoaceticus NCIB8250 isolated by previous workers	51
2	Mutants isolated from A. calcoaceticus strains D4OE and 41	52
3	Mutants isolated from A. calcoaceticus strain C48	54
4	Characterization of strains of A. calcoaceticus	55
5	Summary of the conditions used in this work and elsewhere to enrich mutants of strain NCIB8250 and NF1408 able to grow on D- or L-mandelate	66
6	The effect of growth temperature on D- and L-mandelate dehydrogenase activity	90
7	Effect of current and time of ultrasonic disruption on the release of enzyme activity	102
8	The effect of omitting reagents from the reaction mixture used to measure D-mandelate dehydrogenase	108
9	Variations associated with growing bacteria on different days	114
10	Reproducibility of the disruption and assay procedures	
	(a) Variations due to sonication (b) Variations amongst assays	115
11	Effects of freezing and storing on the activity of D-mandelate dehydrogenase	117
12	Oxygen uptake by a membrane fraction of mutant 219 using D- and L-mandelate as substrates	119
13	Effects of reagents which react with sulphydryl groups on the activities of D- and L-mandelate dehydrogenase	128
14	Effects of metal chelating reagents and related compounds on the activities of D- and L-mandelate dehydrogenase	130
15	Induction of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase in mutant 41 under a range of conditions	134
16	The isolation of mutants of strains D4OE and 41 which constitutively synthesize phenylglyoxylate carboxy-lyase	140
17	Activities of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase in mutants derived from strains 41(a), D4OE(b) and HMM5(c)	141 142 143
18	Activities of the enzymes of the mandelate pathway in various mutants derived from A. calcoaceticus NCIB8250	146

		page
19	Phenylglyoxylate carboxy-lyase activity in mutants isolated from strain 41 which are able to grow well on benzyl alcohol plates	148
20	Frequency of appearance of mutants of strain C48 with ability to grow on L-mandelate	152
21	Activities of the enzymes of the mandelate pathway in mutants derived from strains 61c and C48 which synthesize phenylglyoxylate carboxy-lyase constitutively	155
22	The effect of omitting reagents from the reaction mixtures used to measure D- and L-mandelate dehydrogenase activity for mutants 219 and 364	157
23	Estimation of frequency of mutation of strains NCIB8250 and NF1408 to gain the ability to utilize D-mandelate and of strain NF1408 to gain the ability to utilize L-mandelate	161
24	Summary of the estimation of frequency of mutation of strains NCIB8250 and NF1408 to gain the ability to utilize D-mandelate and of strains NCIB8250 and C48 to gain the ability to utilize L-mandelate	163

SUMMARY

1. This thesis begins with a description of the main attributes of the genus Acinetobacter, drawing attention to its increasing scientific, economic and medical importance. The microbial metabolism of D- and L-mandelate is then reviewed and present knowledge concerning the control of the pathways summarized. The use of metabolic pathways as a means of deducing evolutionary events in microorganisms is then illustrated by a brief discussion of the β-ketoadipate pathway and some comparisons are drawn with the pathways of mandelate metabolism. The mechanisms whereby metabolic capabilities might evolve are then exemplified by a number of experimental models of acquisitive evolution in bacteria. The Introduction concludes with a brief description of a number of mutants used in this work which had been isolated from A. calcoaceticus strain NCIB8250 ($L^{i}D^{o}P^{i}$) by previous workers. This wild-type strain can utilize L-mandelate, but is unable to metabolize D-mandelate. Another wild-type strain EBF65/65 ($L^{o}D^{i}P^{i}$) shows the opposite pattern, utilizing D-mandelate but not L-mandelate.

2. The aims of the experimental work were four-fold:

(a) To define the novel pathway by which D-mandelate is utilized in mutants derived from wild-type strain NCIB8250. Preliminary work by Fewson et al. (1976) had suggested that the initial step involved a specific dehydrogenase, giving phenylglyoxylate which is the first intermediate in the pathway for L-mandelate metabolism. It was hoped either to confirm this or to obtain evidence establishing the existence of some other pathway.

(b) To examine the novel enzyme involved, which turned out to be a D-mandelate dehydrogenase, and compare it with the original L-mandelate dehydrogenase.

(c) To ascertain the mode of regulation of the evolved D-mandelate dehydrogenase in mutants derived from strain NCIB8250 and of the regulation of the evolved L-mandelate dehydrogenase and the original D-mandelate dehydrogenase in mutants derived from wild-type strain EBF65/65.

(d) To attempt to establish the mutational events underlying the appearance of the pathway by which the two wild-type strains of A. calcoaceticus gained the ability to grow on the second stereoisomer of mandelate.

3. A method was developed to overcome the problem of lysis of some mutant strains. Bacteria were grown for a short time in defined medium using a large nutrient broth inoculum. Cultures were grown at 23°C to obtain high specific activity of D-mandelate dehydrogenase because the enzyme was found to be heat-labile.

4. The novel substrate D-mandelate was shown to be stoicheiometrically oxidized to phenylglyoxylate by means of a D-mandelate dehydrogenase. The identity of phenylglyoxylate was determined: (a) spectrophotometrically, (b) by thin-layer chromatography and (c) by characterization of the 2,4-dinitrophenylhydrazone.

5. An accurate, sensitive and reproducible spectrophotometric assay procedure was developed to measure D-mandelate dehydrogenase activity using extracts prepared by ultrasonic disruption of bacteria. The reduction of DCIP was followed at 600nm in the presence of PMS and BSA.

6. Oxygen uptake of an extract or membrane fraction was measured with the oxygen electrode using D- or L-mandelate as substrate. Added DCIP was reduced in preference to oxygen, the reduced form of DCIP

having an apparently inhibitory effect on subsequent oxygen uptake. Analogous behaviour was seen in the presence of PMS, except that the rate of oxygen uptake was about five times greater. The inhibitory effect of reduced DCIP in the presence of PMS was diminished by the addition of BSA. In the absence of PMS, but not in its presence, BSA abolished the preferential reduction of DCIP. With BSA and PMS present, the observed oxygen uptake was stoicheiometrically equivalent to the rate of DCIP reduction measured in the spectrophotometric assay.

7. The novel D-mandelate dehydrogenase activity was partly characterized and shown to be similar to the original L-mandelate dehydrogenase activity in a number of respects:

(a) both enzymes are membrane-bound,

(b) both reduce the electron acceptors DCIP and PMS,

(c) they show complete stereospecificity for their respective substrates,

(d) both are inhibited by the thiol-specific reagents p-chloromercuribenzoate and mercuric chloride,

(e) they are inhibited similarly by low concentrations of oxalate,

(f) they are unaffected by a variety of metal-chelating reagents and related compounds,

(g) they exhibit similar pH optima and temperature-dependence for activity and

(h) they are inhibited by the opposite isomer of mandelate.

8. The novel D-mandelate dehydrogenase in mutant 41 ($L^{i}D^{i}P^{i}$), derived from strain NCIB8250, was found to be co-ordinately regulated with L-mandelate dehydrogenase and phenylgyoxylate carboxy-lyase under various conditions of induction, anti-induction and repression. Furthermore, mutants of strains D4OE ($L^{o}D^{i}P^{i}$) and 41 ($L^{i}D^{i}P^{i}$), isolated

solely on the basis of constitutive synthesis of phenylglyoxylate carboxy-lyase had also acquired the ability to synthesize constitutively the evolved D-mandelate dehydrogenase. Isolation of mutants able to utilize D-mandelate from strain HMM5 ($L^{C}D^{O}P^{C}$) which constitutively synthesizes the original mandelate enzymes, always gave mutants with constitutive D-mandelate dehydrogenase activity. All these observations lead to the conclusion that the evolved D-mandelate dehydrogenase is controlled in a similar manner to the original mandelate enzymes.

9. Mutants with an evolved L-mandelate dehydrogenase (e.g. 61c) were derived from strain C48, an auxotroph of wild-type strain EBF65/65. This novel L-mandelate dehydrogenase was shown to be regulated in a similar manner to the original D-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase since mutants isolated from strain 61c on the basis of the ability to synthesize phenylglyoxylate carboxy-lyase constitutively were also constitutive for both L-mandelate dehydrogenase and D-mandelate dehydrogenase.

10. The evolved and original D-mandelate dehydrogenases in mutants derived from wild-type strains NCIB8250 and EBF65/65 showed similarities in their requirements for PMS and BSA in the spectrophotometric assay and in their relative heat lability. Furthermore, the original and evolved L-mandelate dehydrogenases in these strains were also similar and both were heat-stable.

11. It is proposed that both wild-type strains of A. calcoaceticus possess genetic information for both D- and L-mandelate dehydrogenases. The different wild-type phenotypes with respect to mandelate utilization probably occur because the information for one of the enzymes is not

expressed as an active product. The evolution of the novel mandelate-metabolizing ability would then be due to expression of this 'silent' gene.

I N T R O D U C T I O N

1. Acinetobacter

Acinetobacter is a genus whose taxonomic position has been the subject of considerable disagreement. Attempts to classify these organisms have led them to be grouped into as many as fifteen different genera (Baumann et al., 1968). However, it now appears that Acinetobacter is a well-defined genus which can be clearly differentiated from genera such as Moraxella, Neisseria, Branhemella, Mima, Achromobacter and Herellea to which it was once considered to be very similar and often confused with (Henriksen, 1976; Juni, 1978).

All acinetobacters are aerobic, gram-negative, non-motile [although some strains may move by 'twitching' (Henrichsen & Blom, 1975)], catalase positive, oxidase negative (Kovács) and their DNA composition varies from approximately 38-47mol % G+C (Juni, 1978; see also Table 4 of Methods). The identity of the genus, although it contains many strains sharing a wide variety of properties (see e.g. Baumann et al., 1968), has been confirmed by the specificity of genetic transformation within the genus and by DNA-DNA hybridization experiments (Juni, 1978).

One species, A. calcoaceticus, is generally recognised (Henriksen, 1976) even though the name may not have been validly published (see footnote to Henriksen, 1976). Some taxonomists prefer to divide the genus into two species: A. calcoaceticus and A. lwoffi, largely on the basis of the ability to oxidize carbohydrates.

Acinetobacter strains have a wide distribution in Nature although the main habitats appear to be soil and water (Henriksen, 1973).

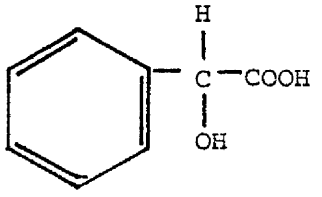
Baumann (1968) estimated that acinetobacters represent at least 0.001% of the total bacterial population capable of aerobic growth in these environments. Acinetobacters are also found associated with humans (Henriksen, 1973), frequently as contaminants from the surroundings. Although not generally considered to be serious pathogens, organisms of this genus have been implicated in a wide variety of infections especially in hospitalised patients (Glew et al., 1977; von Graevenitz, 1977; Rosenthal, 1978). They have usually been found to be contaminants or relatively harmless secondary invaders, but occasionally have caused serious or even fatal infections (Henriksen, 1973). They have also been isolated from farm animals, both as harmless commensals or contaminants and as apparent causes of disease (Henriksen, 1973; Dickie & Regnier, 1978; Rajasekhar et al., 1978). Acinetobacters appear to be unaffected by many commonly used antibiotics and this can cause clinical problems and may explain why they are being found in increasing numbers, especially in hospitals (Glew et al., 1977; Rosenthal, 1978). This genus also appears to be relatively resistant to the ionising radiation used in food preparation and acinetobacters have been found in fish (Lee & Harrison, 1968), poultry (Thornley et al., 1960) and meat products (Tiwari & Maxcy, 1972; Ito et al., 1976) during investigations of food spoilage. Acinetobacters are able to grow on hydrocarbons and this presumably explains why they have also been found associated with oil pollution in aquatic environments (e.g. Gutnick & Rosenberg, 1977; Bartha & Atlas, 1977). The ability of these bacteria to use a wide range of organic compounds as carbon sources suggests that they can accommodate to the availability of a variety of natural and industrial products.

The wide nutritional versatility, frequency of occurrence and possible economic and clinical importance are all reasons why an increasing amount of physiological and biochemical work is being done with A. calcoaceticus (see review by Juni, 1978). The recent developments of systems of genetic transformation (Juni, 1978), conjugation with plasmids such as RP4 (Towner & Vivian, 1976), and transduction (Herman & Juni, 1974; Juni, 1978) should help to complement the biochemical work now being done with this species, and mapping of the apparently circular chromosome has now started (Juni, 1978; Towner, 1978).

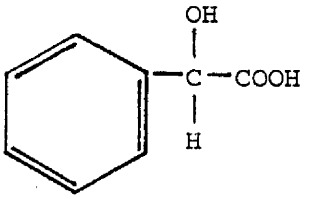
Strain NCIB8250 has been classified as a strain of Acinetobacter calcoaceticus (Véron, 1966; Fewson, 1967a, b; Baumann et al., 1968). This organism is unable to metabolize carbohydrates (Cook & Fewson, 1973) but can utilize a wide variety of organic compounds, including many aromatic compounds, as sole sources of carbon and energy (Fewson, 1967a). The dissimilation of these aromatic compounds is catalyzed by successive converging pathways of inducible catabolic enzymes (Fewson, 1967a; Kennedy & Fewson, 1968a). The complexity of many of these pathways makes their regulation particularly interesting. Work in Glasgow has been centred on the mandelate pathway, a catabolic unit within the system (e.g. Kennedy & Fewson, 1968b; Livingstone & Fewson, 1972; Livingstone et al., 1972; Cook et al., 1975; Fewson et al., 1978).

2. Metabolism of mandelate and related compounds

Mandelic acid (phenylglycolic acid; amygdalic acid) is an aromatic acid of plant origin (Carles & Bourguet, 1957), and both D(-) and L(+) isomers (I and II, respectively) are thought to occur in Nature.



(I)



(II)

Ring-substituted mandelates are almost certainly found as the result of biodegradation of lignin, but there appears to have been no attempts to determine their presence and concentration in soils. Amygdalin (D-mandelonitrile-β-D-glucosido-6-β-D-glucoside), a constituent of seeds such as almonds, although containing the elements of mandelate, is probably broken down directly to benzaldehyde and not to mandelate (see Merck Index, 1976). 3-Methoxy-4-hydroxymandelic acid (vanillylmandelate) and 4-hydroxymandelic acid are excreted by many vertebrates and invertebrates as a result of breakdown of noradrenaline, adrenaline and octopamine (Axelrod & Saavedra, 1977) and so presumably must be found in soil. Mandelic acid is also found in the urine of industrial workers exposed to styrene (vinyl benzene; Otsuji & Ikeda, 1970), and is one of the urinary metabolites of styrene in rabbits (El Marsi *et al.*, 1958).

Mandelate can be used as sole carbon and energy source by some bacteria of the genera *Pseudomonas* (Stanier *et al.*, 1966), *Acinetobacter*

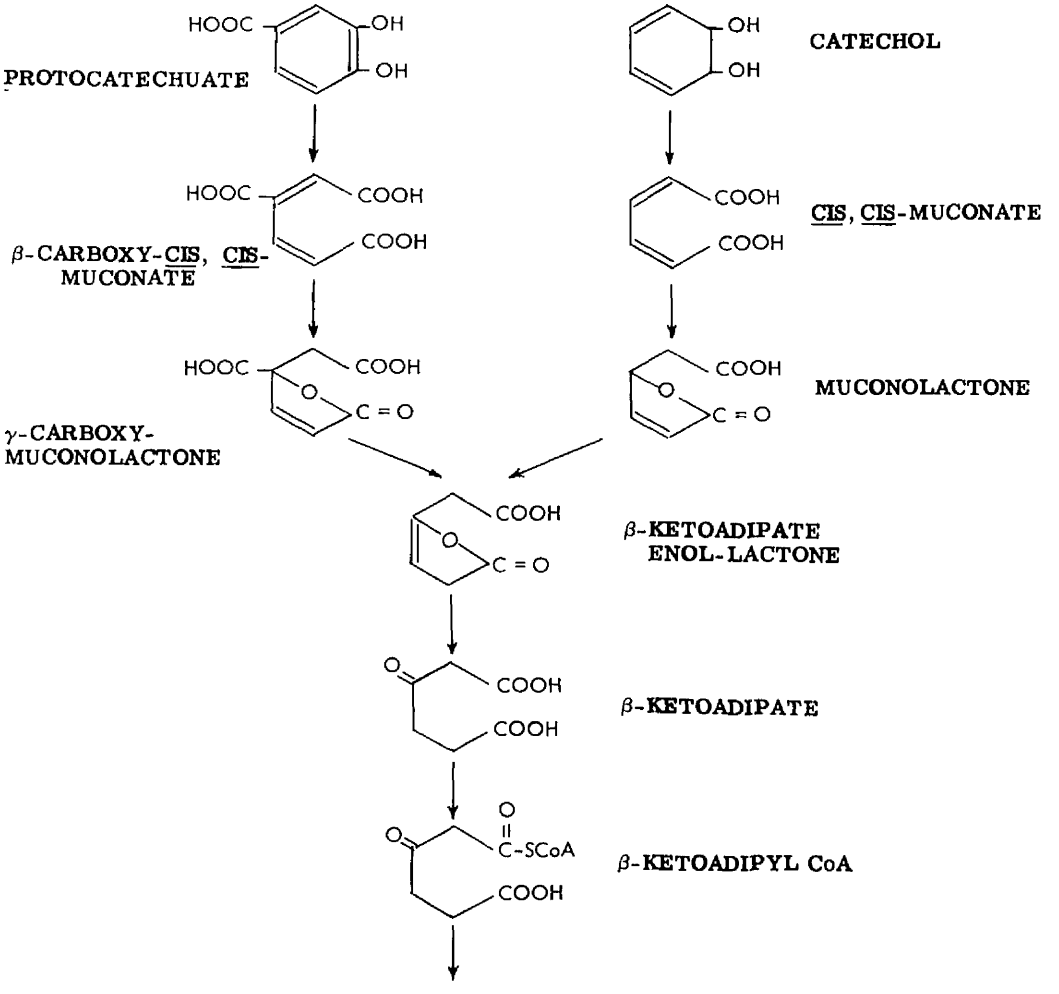
(Baumann et al., 1968), Azotobacter and Bacillus (Hegeman et al., 1970) and by the fungi Aspergillus niger (Jamaluddin et al., 1970), Neurospora crassa (Ramakrishna Rao & Vaidyananthan, 1977) and some yeasts (Hegeman et al., 1970).

The metabolism of mandelate can be considered in two parts:

(a) manipulation of the side chain and ring hydroxylation leading to the formation of catechol or protocatechuate (3,4-dihydroxybenzoate),

(b) ring cleavage of catechol or protocatechuate, with subsequent production of the two important amphibolic intermediates, succinate and acetyl-CoA.

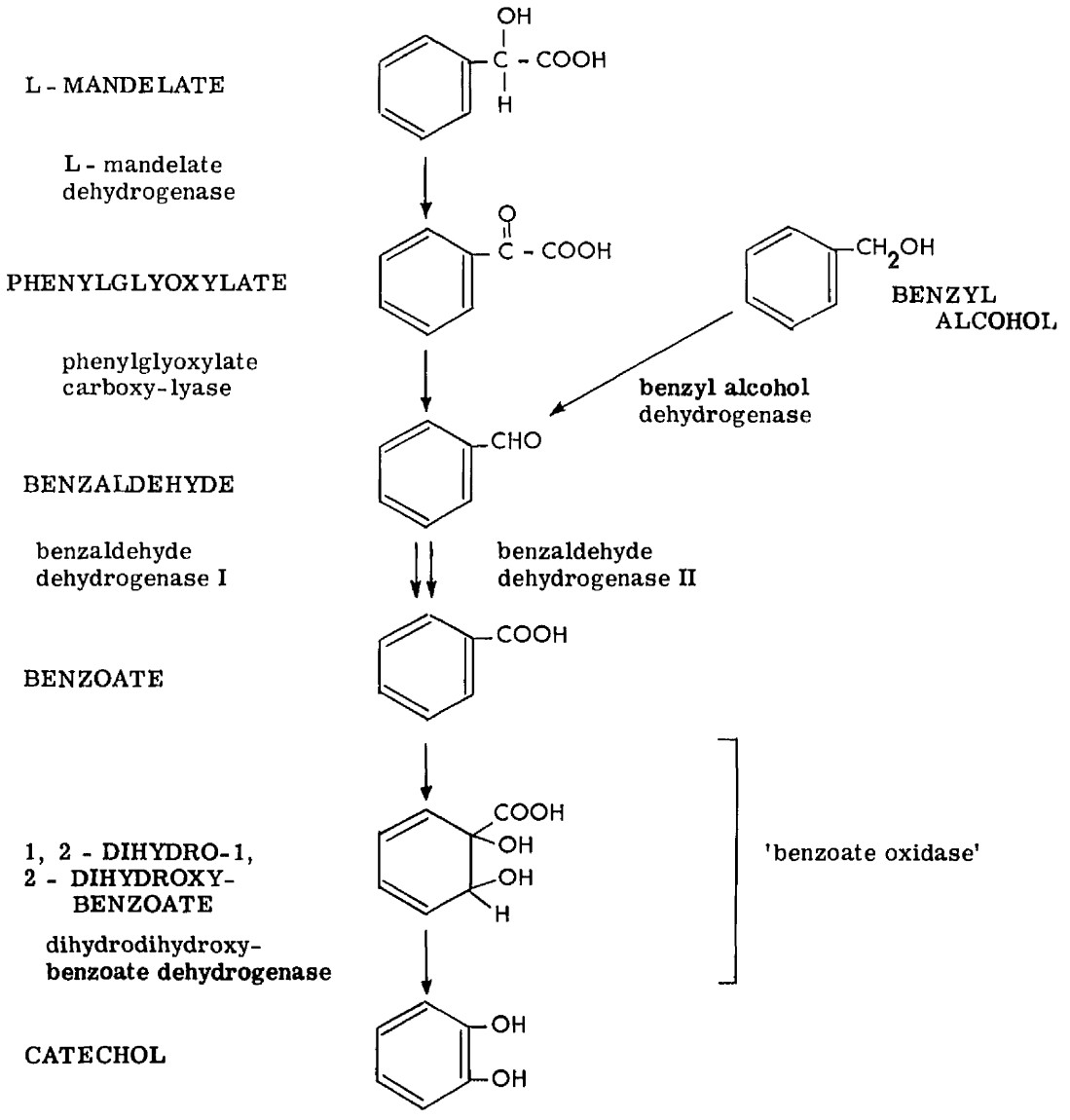
Generally only those processes involved in the first part of mandelate metabolism, i.e. to the level of catechol or protocatechuate, will be considered in detail in this thesis. The reactions involved in the further metabolism of these two compounds, as well as the control of the appropriate enzymes have been extensively reviewed (e.g. Dagley, 1971; Stanier & Ornston, 1973) and will not be discussed here in detail, although the evolution of the pathway will be mentioned in Section 4 of this Introduction. Essentially the metabolism of protocatechuate and catechol involves ortho or meta cleavage of the benzene ring. Scheme 1 gives the intermediates of the pathway as found in all bacteria, studied so far, which metabolize protocatechuate and catechol via the ortho cleavage pathway (often referred to as the β-ketoadipate pathway).



THE β - KETOADIPATE PATHWAY FOR THE METABOLISM OF CATECHOL AND PROTOCATECHUATE IN BACTERIA

Scheme 1

In Acinetobacter calcoaceticus NCIB8250, Kennedy and Fewson (1968a), using techniques of simultaneous adaptation (Stanier, 1947a), showed that L-mandelate was oxidized to catechol via phenylglyoxylate (benzoylformate), benzaldehyde and benzoate (Scheme 2). A convergent pathway degraded benzyl alcohol to catechol via benzaldehyde and benzoate. The following enzyme activities were identified in extracts: L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase, two benzaldehyde dehydrogenases (one heat-stable, benzaldehyde dehydrogenase I; one heat-labile, benzaldehyde dehydrogenase II), benzyl alcohol dehydrogenase (Kennedy & Fewson, 1968b), and 'benzoate oxidase' (Fewson et al., 1970). The benzaldehyde dehydrogenases and benzyl alcohol dehydrogenase require NAD^+, but the natural hydrogen acceptor of L-mandelate dehydrogenase is not known. Phenylglyoxylate carboxy-lyase requires thiamin pyrophosphate as cofactor. Subsequent conversion of benzoate to catechol may involve two or more metabolic steps. In A. calcoaceticus strain 73 and other bacteria (Reiner, 1971; Reiner & Hegeman, 1971; Reiner, 1972) benzoic acid is converted to 1,2-dihydro-1,2-dihydroxybenzoate (3,5-cyclohexadiene 1,2-diol 1-carboxylic acid) by a dioxygenase. This enzyme appears to be unstable and has not been isolated so it is not known if a single protein is involved in this step. Dihydrodihydroxybenzoate dehydrogenase, which converts the diol to catechol, was purified by Reiner (1972) from Alcaligenes eutrophus and shown to be a single enzyme. The enzymes converting benzoate to catechol will be referred to as 'benzoate oxidase' in this thesis. Further metabolism of catechol by A. calcoaceticus NCIB8250 involves ortho ring cleavage to cis,cis-muconate (Kennedy & Fewson, 1968b) which is then metabolized via the β-ketoadipate pathway (Scheme 1; Ornston & Stanier, 1966).



PATHWAY OF L - MANDELATE AND BENZYL ALCOHOL METABOLISM

IN Acinetobacter calcoaceticus NCIB8250

Scheme 2

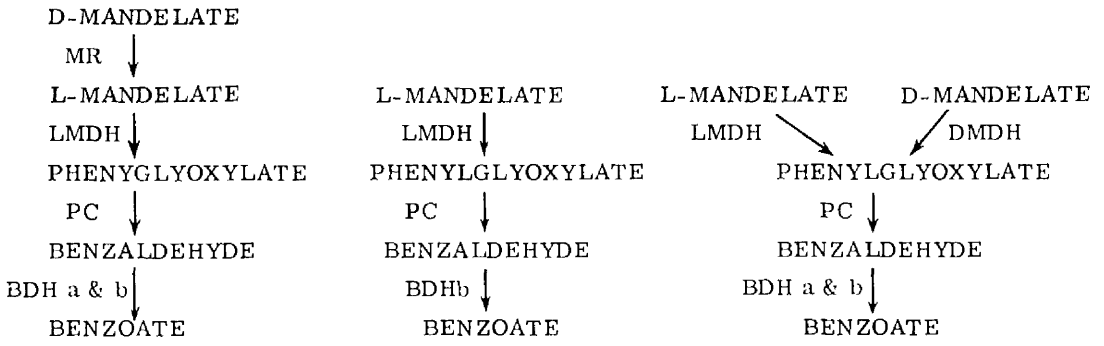
A. calcoaceticus NCIB8250 has a dehydrogenase specific for L-mandelate, and can metabolize L-mandelate, but not D-mandelate. About four years ago Dr C.A. Fewson (unpublished work) showed that the strain EBF65/65 used by Dr A. Vivian for his experiments on genetic recombination (Towner & Vivian, 1976, 1977) shows the opposite pattern; it can metabolize D-mandelate, but has no L-mandelate dehydrogenase activity. Very preliminary work indicated that the rest of the pathway appeared to be similar in the two strains. In a comparative study of 106 strains from the genus Acinetobacter, Baumann et al. (1968) found three which utilized D-mandelate and L-mandelate , one which utilized only the L-isomer (strain NCIB8250), and one strain which utilized phenylglyoxylate but neither mandelate as sole sources of carbon and energy. Dr C.A. Fewson (unpublished work) found that out of 35 strains isolated by Dr E. Barnes (strains obtained from Dr A. Vivian), eight were able to utilize both isomers and one (strain EBF65/65) could utilize the D-isomer only.

Although the mandelate dehydrogenase in A. calcoaceticus NCIB8250 shows stereospecificity, it is relatively non-specific as regards substituents on the ring. The 2-hydroxy, 4-hydroxy, 3,4-dihydroxy and 4-hydroxy-3-methoxy (vanillyl) derivatives of L-mandelate as well as the corresponding benzyl alcohols are converted to the corresponding derivatives of benzoate apparently by the same enzymes that metabolize the unsubstituted compounds (Kennedy & Fewson, 1968a, b). Subsequent catabolism of each hydroxy-substituted benzoate is catalyzed by a specific hydroxylase or oxygenase. 2-Hydroxybenzoate (salicylate), like benzoate, is metabolized to catechol whilst other substituted compounds are converted to protocatechuate.

Pseudomonas putida A.3.12. (ATCC12633, NCIB9494; previously referred to as Pseudomonas fluorescens; strain 90 of Stanier et al., 1966) had been shown earlier to metabolize L-mandelate through the same sequence of metabolic intermediates (Stanier, 1947a, b; Gunsalus et al., 1953a, b; Stanier et al., 1953; Hegeman, 1966a, b, c; Ornston & Stanier, 1966; Cánovas et al., 1967; Scheme 3). P. putida A.3.12. can also utilize D-mandelate by coverting it to L-mandelate by means of a mandelate racemase. This enzyme was first described by Gunsalus et al. (1953b), and since then has been studied in more detail (Weil-Malherbe, 1966; Hegeman et al., 1970; Hegeman & Kenyon, 1970; Fee et al., 1974a, b; Maggio et al., 1975). A strain of P. putida (ATCC17426; strain 49 of Stanier et al., 1966), which resembles P. putida A.3.12. in many respects, also utilizes D-mandelate, but in this case cannot oxidize L-mandelate. According to Hegeman et al. (1970) this strain has a specific D-mandelate dehydrogenase and no mandelate racemase. A further four strains of P. putida (strains 6, 7, 81 and 111 of Stanier et al., 1966) utilize D-mandelate and phenylglyoxylate, but not L-mandelate. It is possible that these strains also have a specific D-mandelate dehydrogenase. No characterization of this D-mandelate dehydrogenase appears to have been carried out.

Weil-Malherbe (1966) showed that P. putida A.3.12. could metabolize 4-hydroxy-, 4-hydroxy-3-methoxy-, and 3,4-dihydroxy-L-mandelate, but 4-hydroxy-D-mandelate was the only substituted D-isomer to be a substrate for the racemase.

P. putida has an NAD^+-linked and an $NADP^+$-linked benzaldehyde dehydrogenase, both associated with mandelate metabolism (Gunsalus et al., 1953a), and a third dehydrogenase appears to be synthesized during growth on benzaldehyde as carbon source (Stevenson & Mandelstam,



PATHWAYS FOR MANDELATE OXIDATION TO BENZOATE IN THREE MICROORGANISMS

Scheme 3

MR	mandelate racemase
LMDH	L-mandelate dehydrogenase
DMDH	D-mandelate dehydrogenase
PC	phenylglyoxylate carboxy-lyase
BDHa	NAD^+-dependent benzaldehyde dehydrogenase
BDHb	$NADP^+$-dependent benzaldehyde dehydrogenase

1965). Furthermore, metabolism of benzyl alcohol also seems to be via dehydrogenation to benzaldehyde as in A. calcoaceticus NCIB8250. This was initially suggested by Stanier (1947b) and further speculated on by Hegeman (1966a). Subsequently Suhura et al. (1969) purified an NAD^+-linked benzyl alcohol dehydrogenase and showed the presence of an NAD^+-linked benzaldehyde dehydrogenase in P. putida strain T2 grown on toluene.

Pseudomonas aeruginosa can oxidize L-mandelate only, although the rest of the pathway is the same as that in P. putida and A. calcoaceticus (Rosenberg, 1971; Scheme 3). There is some evidence for the existence of two $NADP^+$-linked benzaldehyde dehydrogenases in P. aeruginosa (Rosenberg, 1971), although the results based on differential induction and mutational analysis were not conclusive. One of the enzymes appeared to be induced by phenylglyoxylate, and the other by β-ketoadipate.

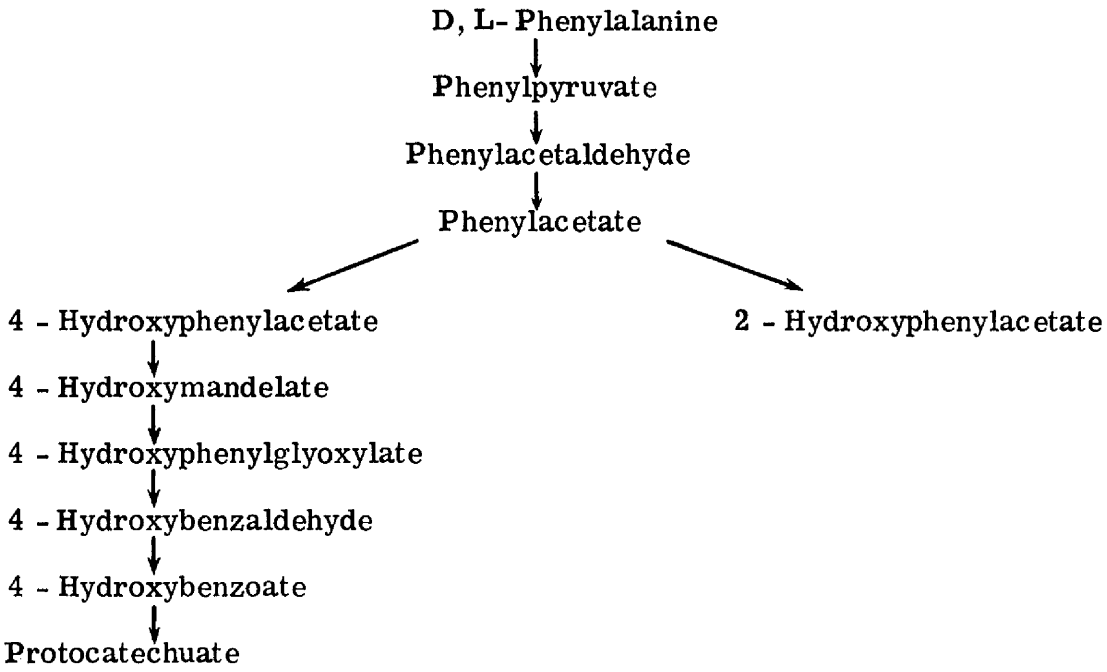
The fungus Aspergillus niger UBC814 utilizes both D- and L-mandelate by means of two stereospecific mandelate dehydrogenases (Jamaluddin et al., 1970; Ramanarayanan & Vaidyanathan, 1973; Scheme 3). The L-mandelate dehydrogenase appears to be soluble (Jamaluddin et al., 1970) in contrast to those of A. calcoaceticus NCIB8250 and P. putida which are 'membrane-bound' and 'particulate' respectively (Kennedy & Fewson, 1968b; Gunsalus et al., 1953; in fact the so-called 'particulate' preparation may reasonably be assumed to indicate a membrane-bound enzyme, see Nakada & Nosu, 1958). Unlike the bacterial enzyme, which does not require any known cofactor, the activity of the fungal dehydrogenase was stimulated by FAD or FMN. The D-mandelate dehydrogenase of A. niger, however, resembled the bacterial L-mandelate dehydrogenase in its particulate nature (probably mitochondrial; private communication

from Dr M. Jamaluddin to Dr C.A. Fewson), lack of stimulation by FAD or FMN and NAD(P)-independence, but differed from the bacterial enzyme in its inability to reduce the artifical electron acceptor 2,6-dichlorophenolindophenol.

In A. niger, two benzaldehyde dehydrogenases, one NAD^+- dependent and one $NADP^+$-dependent, convert benzaldehyde to benzoate (Jamaluddin et al., 1970). The mandelate pathway deviates from the bacterial pathway at the level of benzoate which is converted to 4-hydroxybenzoate by the enzyme benzoate-4-hydroxylase (Reddy & Vaidyanathan, 1975), and further metabolized via the protocatechuate branch of the β-ketoadipate pathway.

D,L-4-Hydroxymandelate was also found to be oxidized by A. niger UBC814 (Jamaluddin et al., 1970), presumably through a parallel pathway to 4-hydroxybenzoate. Thus in A. niger, 4-hydroxybenzoate is the converging point in the metabolism of mandelate and 4-hydroxymandelate, both of which are dissimilated through the protocatechuate pathway. This again is in contrast to the bacterial system, in which mandelate is degraded through the catechol pathway, and only 4-hydroxymandelate is degraded through the protocatechuate pathway, the two pathways converging at β-ketoadipate (Gunter, 1953; Stevenson & Mandelstam, 1965; Kennedy & Fewson, 1968a).

4-Hydroxymandelate has also been shown to be an intermediate in the metabolism of D,L-phenylalanine by a strain of A. niger isolated from soil (Kishmore et al., 1974; Scheme 4). It was reported that 4-hydroxymandelate dehydrogenase activity could be demonstrated in a particulate as well as a soluble fraction, $NADP^+$ being the most effective cofactor of both systems. In contrast Sugurmaran et al. (1973)



PROPOSED PATHWAY FOR PHENYLALANINE DEGRADATION IN A STRAIN OF Aspergillus niger ISOLATED FROM SOIL

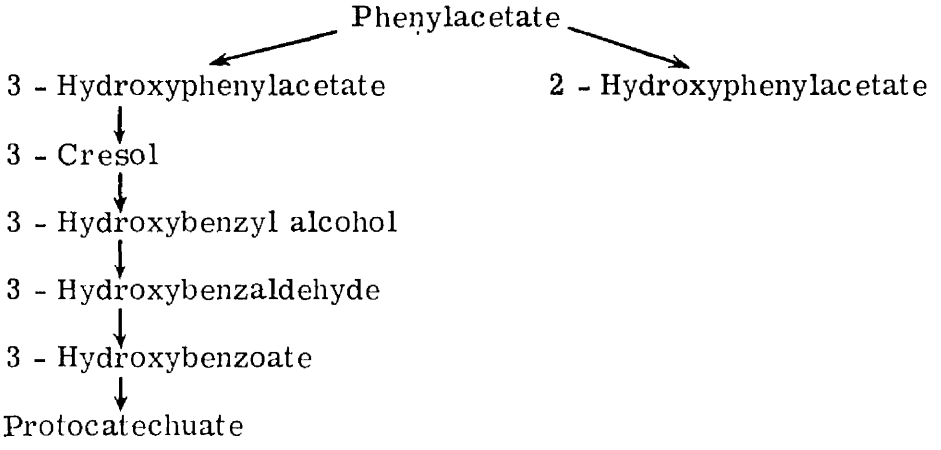
(Kishmore et al., 1974)

Scheme 4

implicated 3-hydroxy-benzylalcohol, 3-hydroxybenzaldehyde and 3-hydroxy-benzoate in the catabolism of phenylacetate by A. niger UBC814 (Scheme 5). An $NADP^+$-linked benzyl alcohol dehydrogenase and an NAD^+-specific benzaldehyde dehydrogenase were found to be associated with this pathway.

The degradation of mandelate in Neurospora crassa follows a similar pathway to that in Aspergillus niger (Ramakrishna Rao & Vaidyanathan, 1977). Both D- and L-mandelate are used by N. crassa presumably either by a racemase or by dehydrogenases specific for the D- and L- enantiomers. Benzoate is converted to 4-hydroxybenzoate, which is metabolized via the protocatechuate branch of the β-ketoadipate pathway.

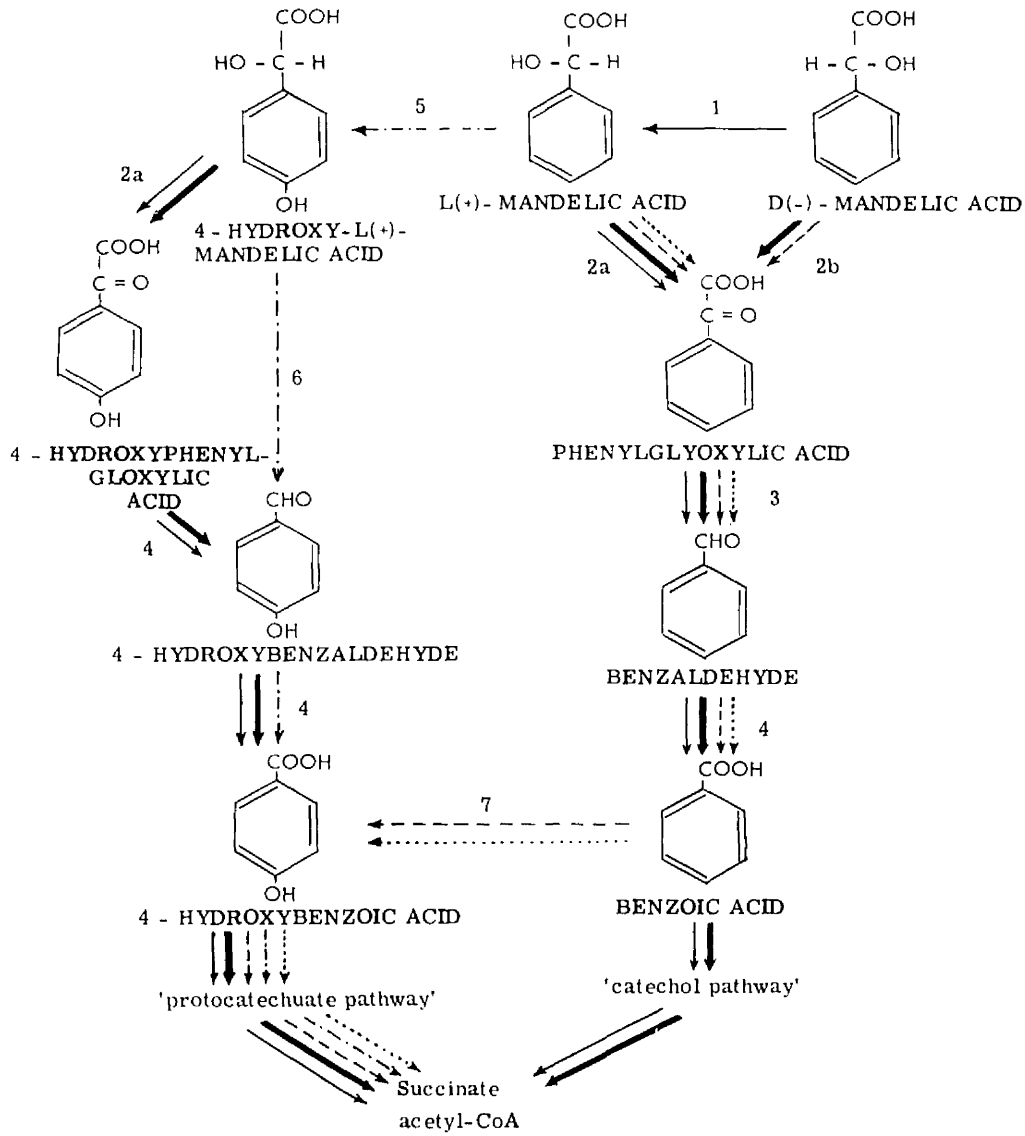
In all the microorganisms discussed so far mandelate is converted to benzoate via phenylglyoxylate and benzaldehyde. 4-Hydroxymandelate is metabolized by an analogous pathway through 4-hydroxyphenylglyoxylate and 4-hydroxybenzaldehyde to 4-hydroxybenzoate, using the same set of enzymes. Pseudomonas convexa, however, appears to differ from other microorganisms in the chemical events of the mandelate pathway. An enzyme, L-mandelate-4-hydroxylase, converting L-mandelate to L-4-hydroxymandelate was reported by Bhat et al. (1973; Scheme 6). This enzyme is very specific for L-mandelate, and will not hydroxylate D-mandelate. It is apparently soluble, requiring tetrahydropteridine, NADPH, Fe^{2+} and O_2 for its activity (Bhat & Vaidyanathan, 1976a). 4-Hydroxymandelate is then converted to 4-hydroxybenzaldehyde by the enzyme 4-hydroxymandelate oxidase (Bhat & Vaidyanathan, 1976b) in a single step with the stoicheiometric consumption of O_2 and liberation of CO_2. This enzyme is membrane-bound and requires FAD and Mn^{2+}



PROPOSED PATHWAY FOR THE DEGRATION OF PHENYLACETATE

BY Aspergillus niger UBC 814 (from Sugumaran et al., 1973)

Scheme 5



Acinetobacter calcoaceticus
Pseudomonas putida
Pseudomonas convexa
Aspergillus niger
Neurospora crassa

1 - Mandelate racemase
2a - L (+) - Mandelate dehydrogenase
2b - D (-) - Mandelate dehydrogenase
3 - Phenylglyoxylate carboxy-lyase (benzoylformate decarboxylase)
4 - Benzaldehyde dehydrogenase (there are several isofunctional enzymes)
5 - Mandelate 4-hydroxylase
6 - 4-Hydroxymandelate oxidase
7 - Benzoate 4-hydroxylase

METABOLISM OF MANDELATE IN VARIOUS MICROORGANISMS

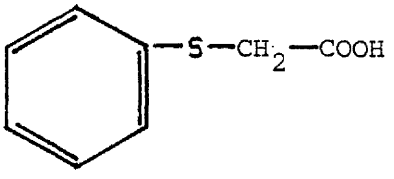
Scheme 6

for its activity. 4-Hydroxyphenylglyoxylate could not be detected as an intermediate in this pathway. In this respect P. convexa differs from the microorganisms previously discussed which all use two consecutive enzymes for this step, i.e. L-mandelate dehydrogenase to convert 4-hydroxymandelate to 4-hydroxyphenylglyoxylate, and then phenylglyoxylate carboxy-lyase to give 4-hydroxybenzaldehyde. 4-Hydroxybenzaldehyde formed in P. convexa is further converted to 4-hydroxybenzoic acid and protocatechuate, and in this respect presents a more familiar pattern.

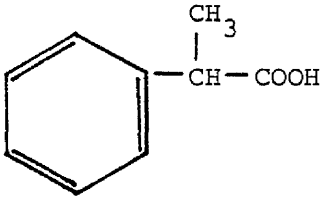
Scheme 6 summarizes the present knowledge of metabolism of mandelate in the various microorganisms.

3. Regulation of the mandelate pathway

In A. calcoaceticus NCIB8250 the first three enzymes of L-mandelate metabolism, L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I, are induced co-ordinately by phenylglyoxylate (Scheme 7; Livingstone & Fewson, 1972) and are referred to as the 'mandelate enzymes' or the 'regulon R_1 enzymes' (a regulon is a group of genes controlling a related function and regulated together by an inducer or repressor, Maas & Clark, 1964). The co-ordinate control of these three enzymes was indicated by: (a) the correlation between the differential rates of synthesis of these enzymes under a variety of conditions including gratuitous induction and repression (Livingstone & Fewson, 1972); (b) the properties of blocked mutants (Livingstone & Fewson, 1972); (c) the gratuitous synthesis of these and no other enzymes by thiophenoxyacetate [(phenylthio) acetate] (Livingstone & Fewson, 1972; III); (d) the co-ordinate anti-induction by 2-phenylpropionate (Fewson et al., 1978; IV);

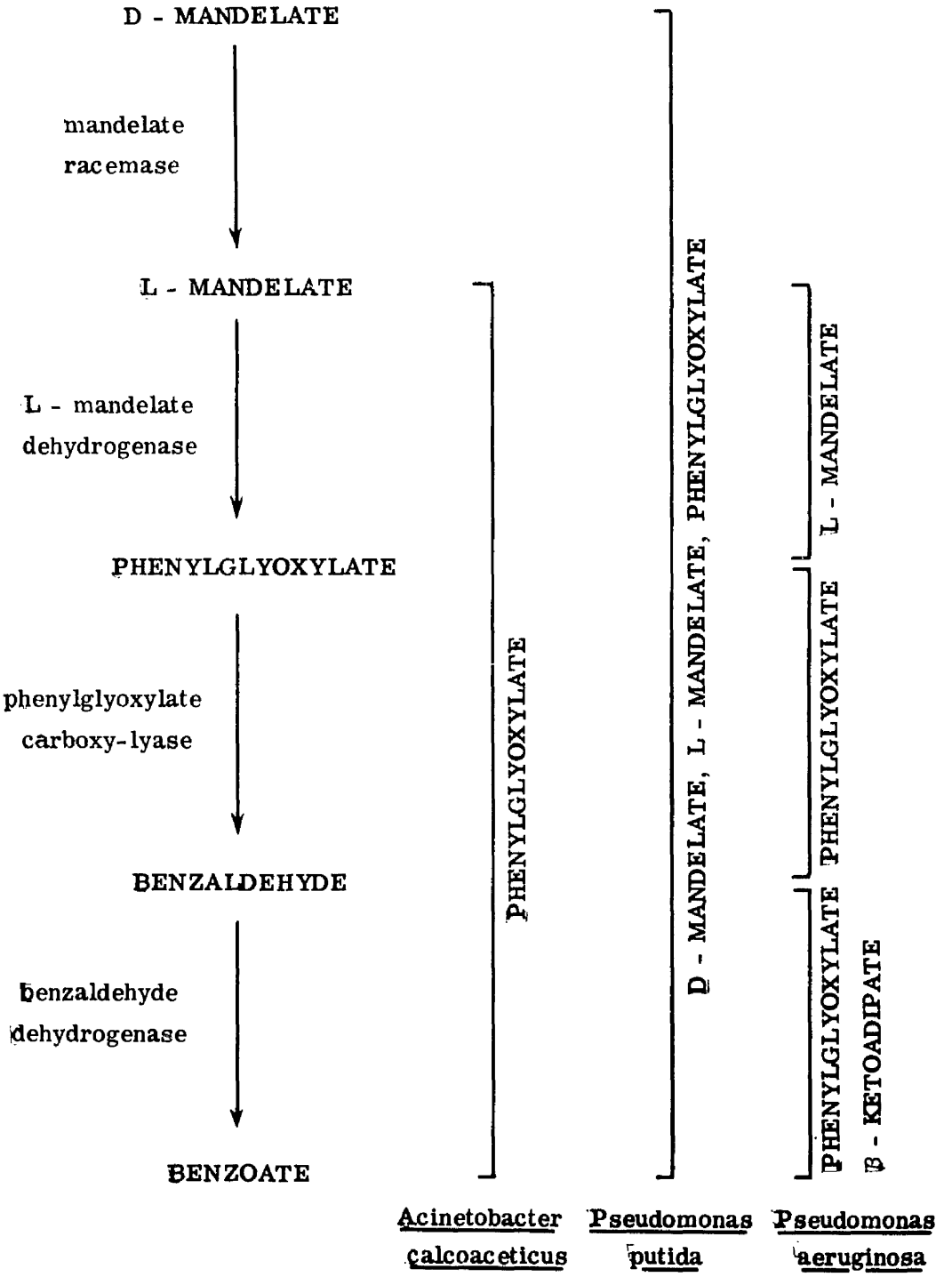


(III)



(IV)

(e) the isolation of mutants selected for constitutive synthesis of phenylglyoxylate carboxy-lyase which all constitutively synthesized L-mandelate dehydrogenase and benzaldehyde dehydrogenase I as well (Fewson et al., 1978). Phenylglyoxylate is the inducer of the regulon



INDUCTION OF ENZYMES FOR THE METABOLISM OF MANDELATE

Scheme 7

since mutant strains lacking phenylglyoxylate carboxy-lyase form the other two enzymes in the presence of L-mandelate or phenylglyoxylate, whereas in mutant strains devoid of L-mandelate dehydrogenase activity only phenylglyoxylate induces phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I (Livingstone & Fewson, 1972).

Results obtained in induction experiments with the wild-type strain NCIB8250 and with mutants showed that benzaldehyde dehydrogenase II and benzyl alcohol dehydrogenase are co-ordinately regulated. They are termed the 'benzyl alcohol enzymes' or the 'regulon R_2 enzymes' (Livingstone et al., 1972). The ability of both benzaldehyde and benzyl alcohol to induce this system is in contrast with the specificity of phenylglyoxylate as inducer of the enzymes of L-mandelate oxidation (Scheme 7).

'Benzoate oxidase' apparently forms a separate regulon (R_3; Livingstone & Fewson, 1972).

Repression of the mandelate enzymes in A. calcoaceticus is observed in the presence of benzoate, catechol and succinate (Cook et al., 1975). The synthesis of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II is repressed by phenylglyoxylate carboxy-lyase, or something synthesized co-ordinately with it, but not by the other mandelate enzymes or by L-mandelate, phenylglyoxylate, benzaldehyde, or benzoate (Beggs et al., 1976; Moyes & Fewson, 1976; Beggs & Fewson, 1977). Benzyl alcohol dehydrogenase appears to be subject to catabolite repression (Beggs & Fewson, 1977).

In P. putida the pattern of enzyme induction is different. The mandelate enzymes are co-ordinately induced by L-mandelate, D-mandelate or phenylglyoxylate (Scheme 7). This conclusion rests upon several

kinds of independent evidence: (a) the response of the wild-type strain to the gratuitous inducer phenoxyacetate (Hegeman, 1966a); (b) the properties of blocked mutants (Hegeman, 1966b); (c) the isolation of mutants which constitutively synthesized enzymes of the mandelate pathway (Hegeman, 1966c). The mandelate pathway is not only controlled by induction, but also by repression (Mandelstam & Jacoby, 1965; Higgins & Mandelstam, 1972a) as in A. calcoaceticus. Repression in P. putida is caused by benzoate, the end product of the action of the mandelate enzymes, and by more distal end products, catechol and succinate. Since the repression mechanism can be activated independently by any one of these repressors, it was called a multi-sensitive repression mechanism (Mandelstam & Jacoby, 1965). The third benzaldehyde dehydrogenase in P. putida is induced separately, apparently by benzaldehyde or 4-hydroxybenzaldehyde (Stevenson & Mandelstam, 1965).

It is not clear whether the anti-induction by 2-phenylpropionate in A. calcoaceticus (Fewson & Foote, 1976; Fewson et al., 1978) represents an example of the so-called multisensitive repression. It is possible that compounds like benzoate or 2-phenylpropionate act by interfering with the interaction of inducer (phenylglyoxylate) with a repressor molecule; this would be consistent with the apparently competitive nature of the processes (Higgins & Mandelstam, 1972a; Fewson et al., 1978).

In both P. putida (Higgins & Mandelstam, 1972a, b) and A. calcoaceticus (Cook & Fewson, 1972b) there is evidence for transport of aromatic compounds by carrier systems; these present possible loci for control. Higgins & Mandelstam (1972a, b) gave several lines of evidence for an inducible transport factor in the active transport of low extracellular concentrations of D,L-mandelate into P. putida. Furthermore the

existence of a permeability barrier to L-mandelate in uninduced bacteria of strain A. calcoaceticus NCIB8250 (Cook & Fewson, 1972b) implies the existence of an inducible system to allow adequate passage of L-mandelate to support mandelate metabolism by intact induced bacteria. The permeability barrier was most pronounced in bacteria grown in benzoate/salts medium; these are conditions under which the mandelate enzymes are known to be repressed (Cook & Fewson, 1972a). The failure of Hegeman (1966b) and Higgins & Mandelstam (1972b) to detect a permeability barrier to mandelate in non-induced bacteria of P. putida may be due either to a difference between the two organisms or to the fact their bacteria possessed low levels of transport factor.

The mandelate pathway enzymes in P. aerugunosa show less extensive co-ordination (Rosenberg, 1971). The first enzyme, L-mandelate dehydrogenase, is induced by its substrate. The second and third enzymes, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase, are induced by phenylglyoxylate (Scheme 7). The same benzaldehyde dehydrogenase, or one very similar, may be induced by β-ketoadipate. These conclusions were deduced from physiological and genetic properties of the wild-type and mutant strains of P. aeruginosa (Rosenberg, 1971).

In A. niger and N. crassa the enzymes of the mandelate pathway are induced on exposure to D,L-mandelate (Jamaluddin et al., 1970; Ramakrishna Rao & Vaidyanathan, 1977), but the detailed induction response of these organisms has not been deduced.

4. Comparison of the evolution and regulation of the β-ketoadipate and mandelate pathways

The β-ketoadipate pathway for the metabolism of catechol and protocatechuate is also controlled by induction and repression. The widespread occurrence of this pathway in both fungi (Cain et al., 1968) and bacteria (Stanier & Ornston, 1973) led to its use as a model system through which the evolutionary relationships between various species and the evolution of metabolic pathways could be inferred. Such studies suggest that the pathway evolved independently in bacteria and fungi since the pathway diverges at the level of β-carboxy-cis,cis-muconate. In bacteria lactonization of this compound gives γ-carboxymuconolactone (see Scheme 1), whilst fungi lactonize it to β-carboxymuconolactone which is then directly converted to β-ketoadipate (Cain et al., 1968). This is reminiscent of the differences in the mandelate pathway at the level of benzoate (Scheme 6). The converging point of mandelate metabolism in A. calcoaceticus, P. putida and P. aeruginosa is benzoate, which is dissimilated through the catechol pathway, whilst 4-hydroxybenzoate is the converging point in the metabolism of P. convexa and the fungi A. niger and N. crassa. Further metabolism of 4-hydroxybenzoate then proceeds via the protocatechuate branch of the β-ketoadipate pathway. In contrast to the difference between bacteria and fungi, in all bacteria so far studied the reactions of the ortho pathway for the metabolism of catechol and protocatechuate are identical (Ornston & Stanier, 1966; Ornston & Parke, 1977). This suggests a common evolutionary origin which contrasts with the differences found in the reactions of the mandelate pathway in P. convexa as compared to A. calcoaceticus, P. putida and P. aeruginosa (Scheme 6; Bhat et al., 1973; Bhat & Vaidyanathan, 1976b).

Although the intermediates of the β-ketoadipate pathway in bacteria are similar, the regulation of the pathway in Acinetobacter and Pseudomonas, for example, is different (e.g. Stanier & Ornston, 1973; Ornston & Parke, 1977). Differences are found in the nature of metabolic inducers and the degree of co-ordinate control. This is comparable with the different regulatory patterns seen in the mandelate pathway in these bacteria (Scheme 7). The induction mechanism of the β-ketoadipate pathway in A. calcoaceticus also requires the synthesis of isofunctional enzymes. For example there are two structurally distinct enzymes that catalyze the hydrolysis of β-ketoadipate enol-lactone. One of these isofunctional enzymes, β-ketoadipate enol-lactone hydrolyase I, is induced with the enzymes of the protocatechuate branch, and the other, β-ketoadipate enol-lactone II, is induced by the enzymes of the catechol branch. Again this compares with the two isofunctional benzaldehyde dehydrogenases in A. calcoaceticus which show no co-ordinacy (Scheme 2; Livingstone et al., 1972).

Although the patterns of induction governing the enzymes of the β-ketoadipate pathway differ, Ornston & Parke (1977) suggested that there may be some homology between the regulatory genes in Acinetobacter and Pseudomonas. The homology could be attributed to either evolution of the β-ketoadipate pathway prior to the divergence of the two genera or to the transfer of genes at a time following divergence. These transfered genes could subsequently evolve to provide the differences now seen between the pathways. Additional evidence favouring the conclusion that the genes for the pathway in the two distinct bacterial species share a common ancestry

comes from a study of the terminal amino acid sequence of the enzyme muconolactone isomerase (Yeh et al., 1978). This enzyme converts muconolactone to β-ketoadipate enol-lactone, and when the enzyme in A. calcoaceticus was compared with that in P. putida, similar sequences were found. In addition the NH_2-terminal amino acid sequence of Acinetobacter enol-lactone hydrolase II (the next enzyme of the pathway) was found to show homology with the Acinetobacter and Pseudomonas muconolactone isomerases. If the observed homology extends over the entire sequence of the proteins, the conclusion may be drawn that a single ancestral enzyme gave rise to the isomerase and hydrolase (Yeh et al., 1978).

Analogous studies applied to the properties and regulation of the enzymes involved in mandelate metabolism seem likely to yield equally informative results concerning the evolution of metabolic capabilities. It is now clear that the chemical intermediates and regulation of this pathway show at least as much variety as the β-ketoadipate pathway, but so far there has been less speculation about evolutionary relationships within the mandelate pathway. Furthermore, as mandelate catabolism lies at the periphery of metabolism, it is likely to provide a better experimental system than the β-ketoadipate pathway since it is more likely to evolve the capability to utilize novel substrates (e.g. Hegeman & Rosenberg, 1970; Clarke, 1974; Wu, 1978).

5. Experimental evolution in bacteria

The deduction of evolutionary relationships from a comparison of metabolic pathways in different organisms is a specific example of the first general approach to the study of evolution as described by Hegeman & Rosenberg (1970):

(a) one can select a group of different organisms and study the relations of a character or constellation of characters within the group and attempt to define the pressures that act upon them,

(b) or one can select a single organism and study the change or occurrence of a character or group of characters produced by the controlled application of selective pressures.

Bacteria are ideal subjects for application of this second experimental approach, as the large populations obtainable and rapid growth allow detection of individually rare events. It is also possible to increase the rate of appearance of mutants by the use of suitable mutagenic treatment and further it is often easy to apply strong selective pressures in one way or another. Usually such 'directed laboratory evolution' (Clarke, 1974) has consisted of exposing a bacterial population to a novel substrate and isolating any mutant strains able to grow. Such studies have revealed a number of mechanisms whereby bacteria acquire new metabolic potential.

There are several reasons why a particular compound may be prevented from being used as a substrate for growth (e.g. see Clarke, 1974):

(a) if it is unable to enter the bacteria,

(b) if there are no enzymes which can convert it to suitable metabolic intermediates,

(c) if the enzymes are not induced by the compound,

(d) if enzymes which can attack the compound, or its products, do so at a rate too low to be effective,

or (e) if the compound, or a product of its metabolism, is an inhibitor of essential cellular activity.

Mutations which affect one or more of these properties may allow growth on a compound which cannot be metabolized by the parent strain.

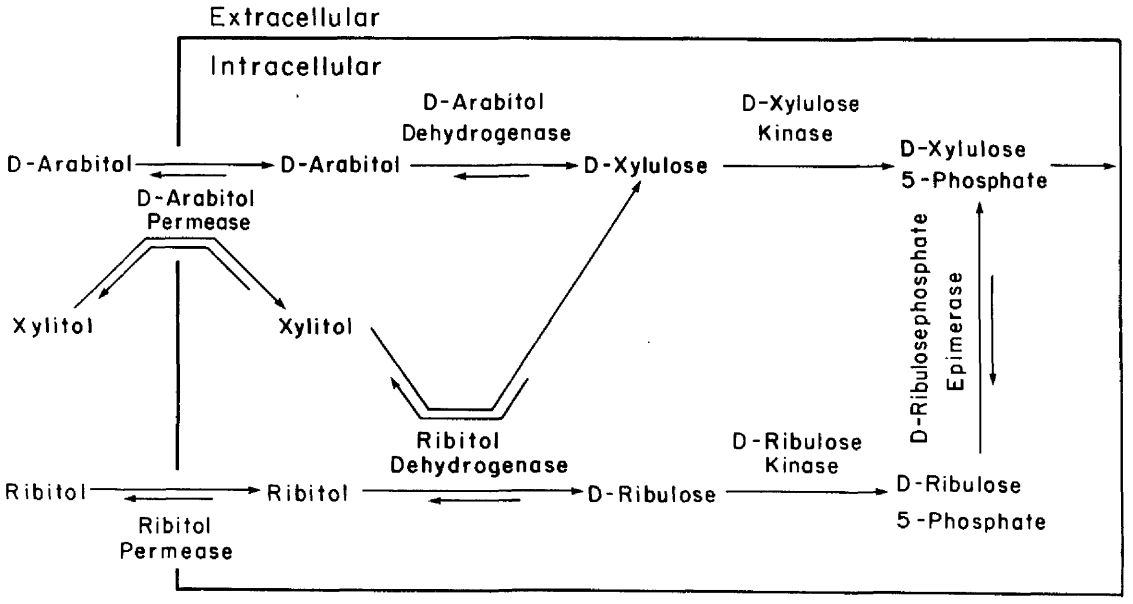
The study of experimental evolution of bacteria has been extensively reviewed in recent years (e.g. Hegeman & Rosenberg, 1970; Clarke, 1974; Jensen, 1976; Riley & Anilionis, 1978; Wu, 1978) and only a few examples will be considered here in order to illustrate the main ways in which metabolic evolution can occur in bacteria.

The specificity of an enzyme is seldom absolute and often a novel compound may be used as a substrate, although usually poorly. In such an instance, growth on the novel substrate may only require sufficient synthesis of the appropriate enzyme. Usually, this has been found to occur by mutation to constitutive enzyme synthesis, rather than by the enzyme becoming inducible by the novel substrate. Presumably this is because mutations leading to constitutivity may not have to be at specific sites, since all that is required is a change which impairs repressor function.

Examples of mutation leading to constitutive enzyme synthesis have been found in Klebsiella aerogenes. This bacterium possesses inducible enzymic pathways to permit it to degrade three aldopentoses (D-ribose, D-xylose, L-arabinose) and two pentitols (ribitol and D-arabitol). Mutants have been selected for growth on many of the less common pentoses and pentitols including D-arabinose, L-xylose,

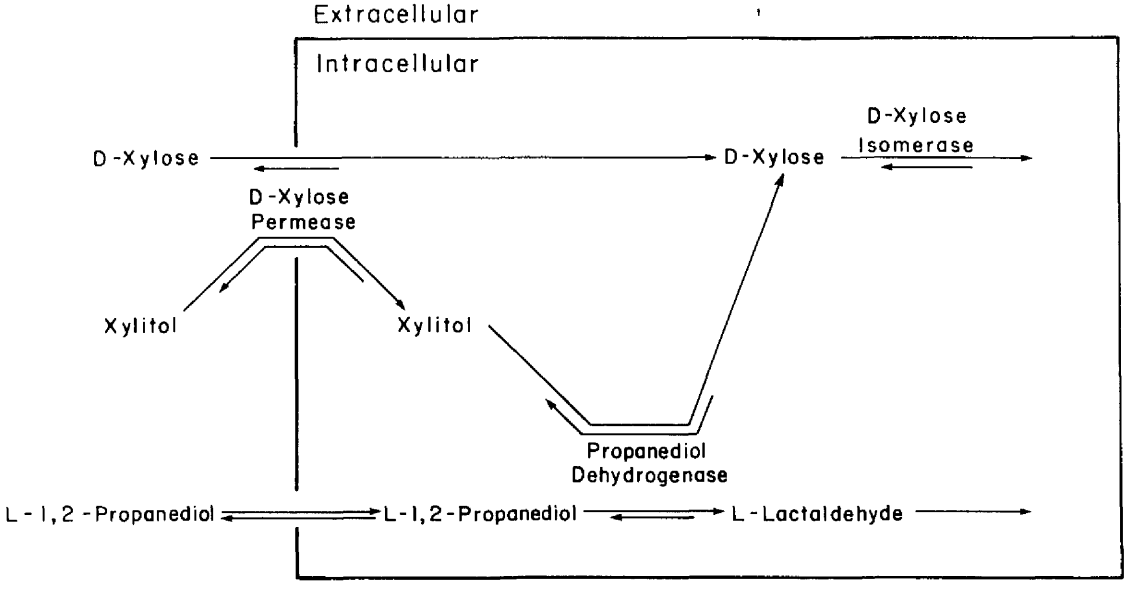
xylitol and L-arabitol. In each case the mutant possesses the necessary enzyme activity to convert the new substrate to a structure which is an intermediate in an existing catabolic pathway (Camyre & Mortlock, 1965; Lerner et al., 1964; Mortlock et al., 1965).

Growth on xylitol and L-arabitol in K. aerogenes strain PRL-R3 was made possible by a mutation to the constitutive synthesis of ribitol dehydrogenase (Mortlock et al., 1965). This work was confirmed by Lerner et al. (1964) with strain 1033. Here a mutant (X1), constitutive for ribitol dehydrogenase synthesis also catalyzed the oxidation of xylitol to D-xylulose, an intermediate in the D-arabitol pathway, although ribitol dehydrogenase usually converts ribitol to ribulose (Scheme 8). This system is further complicated since xylitol is transported by a D-arabitol permease (Wu et al., 1968). Since induction of the permease depends upon a sufficient intracellular concentration of D-xylulose, the initial product of xylitol oxidation, Wu et al. (1968) interpreted the sharp transition in growth rate when the xylitol concentration was increased as the point at which the initial rate of xylitol entry was sufficient to produce inducing concentrations of D-xylulose and hence induce the permease. Thus the new catabolic pathway for xylitol in strain X1 recruits enzymes belonging to two metabolic systems (Scheme 8). The more rapid utilization of xylitol by a second-stage mutant of X1 depended upon a mutation for constitutive synthesis of D-arabitol permease (Wu et al., 1968). The isolation of this latter mutant illustrates the importance of permeation in the evolution of new metabolic capabilities.



METABOLISM OF XYLITOL IN A MUTANT OF Klebsiella aerogenes 1033 (from Wu, 1978)

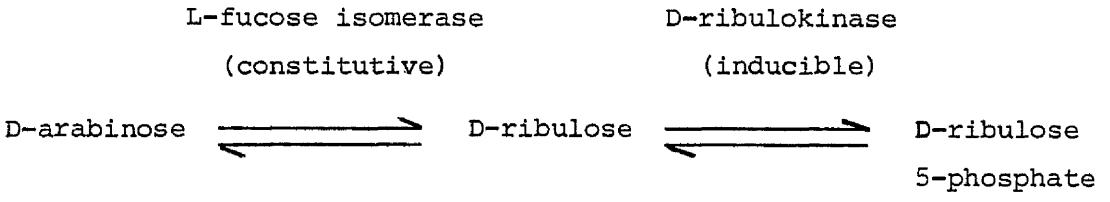
Scheme 8



METABOLISM OF XYLITOL IN A MUTANT OF Escherichia coli K12 (from Wu, 1978)

Scheme 9

Camyre and Mortlock (1965) selected mutants of K. aerogenes for the ability to grow on D-arabinose and found that such mutants were constitutive for L-fucose isomerase. In one mutant, 502, this isomerase apparently converted D-arabinose to D-ribulose which induced D-ribulokinase, an enzyme of the ribitol pathway (Oliver & Mortlock, 1971a), converting D-ribulose to D-ribulose 5-phosphate.



Examples of the utility of constitutive mutants have also been found in bacteria such as Pseudomonas aeruginosa and Escherichia coli.

Using P. aeruginosa 8602, Brown & Clarke (1970) isolated mutants able to use butyramide as a growth substrate. One group of these mutants possessed mutations in the amidase regulator gene allowing them to synthesize large amounts of amidase in the presence of butyramide.

Studies on the evolution of a new lactase function in E. coli have shown that in strains of E. coli K12 bearing a deletion in the lac Z gene (the gene for β-galactosidase) there exists a locus called ebg which may, under sufficiently strong selective pressure, evolve so that its product permits growth on lactose as sole source of carbon (Campbell et al., 1973; Hall & Hartl, 1974). In the first such ebg$^+$ strains described, synthesis of the ebg enzyme was constitutive (Campbell et al., 1973). The identity of the wild-type ebg function is not known, but Arraj & Campbell (1975) suggest it may be metabolism

of a monosaccharide, possibly a 2-amino sugar.

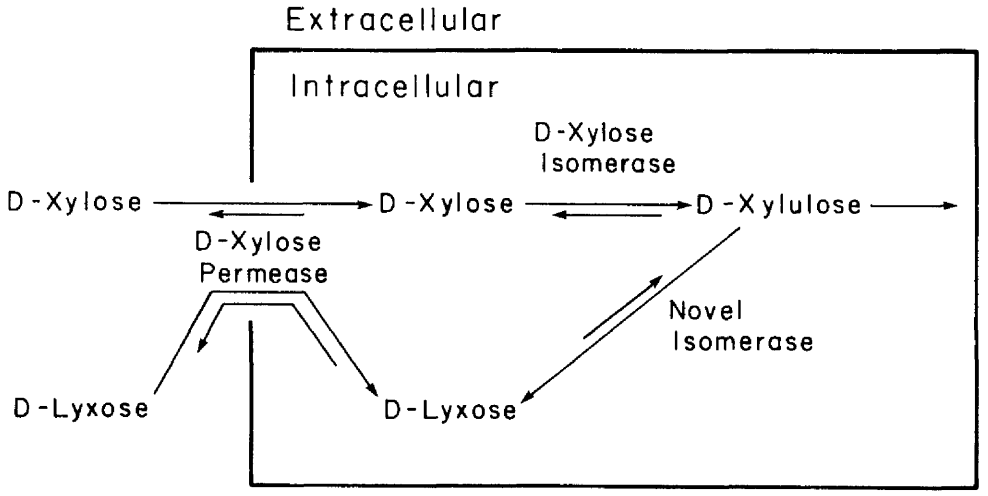
The work of Sridhara et al. (1969) provided the start of a more complex study of experimental evolution. Using E. coli K12 they isolated a mutant able to grow on the novel substrate L-1,2-propanediol, apparently due to constitutive synthesis of a propanediol dehydrogenase. Subsequently this dehydrogenase was shown also to fortuitously convert xylitol to D-xylose, in vitro (Wu, 1976a). As D-xylose can be catabolized by E. coli via xylulose, it was expected that this mutant would grow on xylitol, despite the failure to obtain xylitol mutants by direct selection, even with heavy mutagen treatment. This expectation was not realized directly, but subsequent selection gave a number of xylitol-utilizing mutants, also constitutive for D-xylose permease. It seems that this D-xylose permease transports the xylitol into the bacteria where the constitutive L-1,2-propanediol dehydrogenase converts it to D-xylose, which is metabolized normally (Scheme 9; Wu, 1976a); this provides another example showing the importance of suitable permeation mechanisms.

In the above cases, new functions have arisen by utilizing existing systems for slightly different substrates. A few examples exist, however, where totally new enzymes have appeared. In such cases, it has been postulated that these enzymes were in use at some considerably earlier time but have become inactive or 'silent' in the present organisms. Perhaps the functions are no longer required or have been superseded by a more efficient system. However, if such a 'silent' gene has an appropriate activity towards a novel substrate it may be possible to select for synthesis of the 'silent' gene product.

Wu (1976b) and Stevens & Wu (1976) studied the acquisition of D-arabitol and D-lyxose metabolizing ability in E. coli K12.

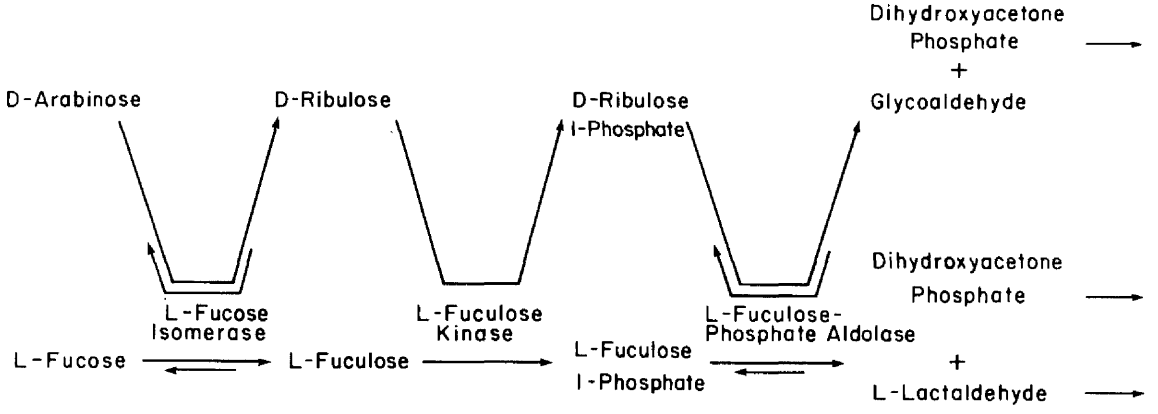
A mutant, 805, constitutive for an isomerase converting D-lyxose to D-xylulose, an intermediate of D-xylose metabolism, was isolated by Stevens & Wu (1976). The D-lyxose apparently enters the bacterium by the D-xylose permease, which is itself induced by the product of D-lyxose isomerization, D-xylulose (Scheme 10). The isomerase proved inactive on related sugars with the exception of D-mannose, which was converted to D-fructose and had a lower K_m (80mM) than that of D-lyxose (300mM). Stevens & Wu (1976) concluded that this isomerase had become 'silent' when the present highly efficient pathway of mannose metabolism, i.e. the phosphofructotransferase system, was evolved. Possibly the original enzyme was more efficient than the K_m of 80mM suggests, since such a 'silent' gene would probably accumulate mutations (see later; p.39).

In the second case a mutant was found to synthesize a dehydrogenase converting D-arabitol to D-xylose (Wu, 1976b). The native substrate may be D-galactose. This mutant was in fact a second-stage mutant isolated from a mutant able to grow on L-1,2-propanediol as described above (Sridhara et al., 1969). It appears that D-arabitol is transported into the bacteria by the L-1,2-propanediol transport system, converted to D-xylulose by the evolved dehydrogenase, and then further metabolized by the D-xylose catabolic system. Again it appears that galactose dehydrogenase is an evolutionary remnant in E. coli, rendered useless by the emergence of a more efficient system for galactose metabolism, i.e. phosphorylation by a kinase to D-galactose 1-phosphate. However, it will be essential to establish that these two enzymes are indeed evolutionary remnants and have no other function at present.



METABOLISM OF D-LYXOSE IN A MUTANT OF Escherichia coli K12 (from Wu, 1978)

Scheme 10



METABOLISM OF D-ARABINOSE BY L-FUCOSE ENZYMES IN A MUTANT OF

Escherichia coli K12 (from Wu, 1978)

Scheme 11

These examples indicate that derepression can be an effective mechanism for evolving new metabolic capabilities. Unless these capabilities are continually used, however, the constitutive synthesis of protein represents a waste of energy and resources. Despite this, derepression is probably the most common mechanism by which bacteria acquire the ability to grow on a novel substrate (Wu, 1978).

In addition to the constitutive mutants described above, other mutants have been isolated in which the novel substrate is able to act as an inducer of a pathway which, fortuitously, metabolizes this substrate. An example of this was found by Leblanc & Mortlock (1971) with *E. coli* K12. They isolated a mutant, 1102, able to grow on the novel substrate D-arabinose which was metabolized by the L-fucose pathway. The initial enzymes of this pathway, L-fucose isomerase, L-fuculokinase and L-fuculose 1-phosphate aldolase were able to convert D-arabinose through D-ribulose to D-ribulose 1-phosphate (Scheme 11). In this mutant, both D-arabinose and L-fucose were able to induce these enzymes, the wild-type being induced by L-fucose only.

Another example of altered induction specificity was provided by Brammer *et al.* (1967) who isolated from succinate + formamide medium, six mutants of *P. aeruginosa* in which amidase synthesis was induced by formamide, which is a poor inducer in the wild-type.

When strains having regulatory mutations enabling growth on a novel substrate are further selected, strains showing better growth may arise due to structural gene mutation. Such mutants alter the affinities of enzymes for substrates, change rates of reaction or modify catalytic activities.

In E. coli K12, a mutant 502, constitutive for L-fucose isomerase synthesis was able to utilize the novel substrate D-arabinose, as previously described. Further selection gave an isomerase having a lower K_m for D-arabinose (Oliver & Mortlock, 1971b).

Again, mutant X1 having a constitutive ribitol dehydrogenase supporting growth on xylitol, was subjected to further selective pressure, and a faster growing mutant, X2, was obtained after NTG treatment. This mutant enzyme was more heat-labile, suggesting a structural gene mutation (Lerner et al., 1964). Rigby et al. (1974) compared the original enzyme of mutant X1 (renamed A) and the altered ribitol dehydrogenase of X2 (renamed B). The enzymes were purified and it was shown that the maximum velocity of the enzyme from X1 (or A) on xylitol was only 12% of that on the natural substrate ribitol, whilst the maximum velocity on xylitol had doubled in the enzyme from X2 (or B), without any significant loss of activity for ribitol. A large improvement (two to four fold) in binding of xylitol to the enzyme from strain X2 was also observed. These results were interpreted in terms of a model in which the new enzyme in X2 results from a small change of amino acid sequence and conformational equilibrium of the molecule. Hartley (1974) concluded that there is no way in which the efficiency of ribitol dehydrogenase can be improved by a single-step amino acid mutation. The extent to which the ribitol dehydrogenase can be evolved appears to be limited. This is in contrast to the Pseudomonas amidase mutants obtained by Clarke & co-workers (see Clarke, 1974) in which a whole new galaxy of enzyme specificities appear by point mutations.

A number of mutants having altered amidases were isolated using amidase regulator mutants and applying selective pressure by providing a variety of novel amides (Betz et al., 1974). In an extreme case, a mutant amidase able to utilize N-phenylacetamide was shown to differ from the wild-type amidase in a single amino acid residue; a threonine residue of the wild-type was replaced by an isoleucine in the mutant enzyme (Brown & Clarke, 1972). This mutant amidase was more heat-labile, and retained about 20% of the specific hydrolase activity for acetamide possessed by the original enzyme.

Structural gene modification provides a means of improving the rate of utilization of novel substrates, but is not the only mechanism possible. Another possibility is to synthesize more of the same enzyme. Although initial utilization of a novel substrate often involves synthesis of the desired enzyme by means of constitutivity or altered inducibility, as described above, increased synthesis may be obtained by promoter mutations, catabolite derepression or gene amplification.

Nicotinamide deamidase in E. coli is active in a cyclical salvage pathway for the production of NAD from nicotinamide. This enzyme is microconstitutive, capable of hydrolyzing about 3nmol nicotinamide min^{-1} $(mg\ protein)^{-1}$. Mutants which were isolated after NTG treatment, were hypoconstitutive for the deamidase, producing sufficient enzyme for the mutants to be able to use nicotinamide as nitrogen source. These mutants were thought to have more efficient promoters (Pardee et al., 1971). Some P. aeruginosa amidase mutants isolated by Smyth & Clarke (1975) also appear to be promoter mutants. The amidase mutation permits growth on butyramide + succinate. The amide is a very poor substrate, and normally represses amidase synthesis, whilst the carbon source, succinate, is a very potent catabolic repressor.

Promoter mutations and gene amplification were reported during further work on the xylitol utilizing mutant of K. aerogenes (X1 or A) isolated by Lerner et al. (1964). Rigby et al. (1974) were able, by continuous culture techniques, to isolate a mutant A1 with constitutive levels of ribitol dehydrogenase about five times that of A (X1). Selection of this mutant gave A11 with fifteen times the enzyme activity of A. Eventually mutants possessing 20% total soluble protein as ribitol dehydrogenase were obtained. By measuring the frequency at which ribitol dehydrogenase activity was lost they concluded that the step from A1 to A11 probably involved gene multiplication, whilst the step from A to A1 probably involved a promoter mutation. The chief evidence for this idea was the observation that segregation of strain A11 gave strains having low levels of ribitol dehydrogenase at a rate of about 0.14%, whereas no segregation was observed in strain A1.

These conclusions were supported by Inderleid & Mortlock (1977) using another K. aerogenes strain, W70. They have shown that only the ribitol dehydrogenase gene was duplicated but none of the other genes of either the ribitol or D-arabitol pathway (which are cotransduced at a high frequency with the ribitol dehydrogenase gene by phage PW52) were duplicated. Furthermore, their results suggested that expression of these duplicated genes was modulated by a catabolite repression system. Despite catabolite repression all gene copies are retained although whether repression acts on all or only some of them is not known. The finding of gene duplication in such investigations is consistent with the suggestions of Koch (1972) who, on theoretical grounds, proposed that duplicate gene copies would be rapidly established in a population in which growth was limited by the gene product. If conditions changed so that this gene product was no longer limiting then the transcription and translation of these duplicate genes would

constitute a metabolic 'burden' to the bacterium placing it at a selective disadvantage relative to those bacteria possessing only a single copy. For this reason, such duplicates would eventually become lost from the population. The relative time scale for these events appears considerable, however. Koch estimated that whilst bacteria possessing duplicated genes could constitute half of the population within a few weeks of selection being applied, the loss of such duplicates would take several years. Significantly, if these gene duplicates mutate so that either translation of the gene product or transcription of the gene itself becomes reduced (thus reducing the burden of the duplicate gene to the bacterium), then loss of duplicated genes would take considerably longer, possibly in the order of thousands of years. Such changes could involve promoter or polar mutations, for example, and would produce genes which were 'silent' in the sense that no active gene product would be formed (although fragments of the original gene product might still be translated).

Hartley (1974) and Koch (1972) speculated that mutations could accumulate in 'silent' genes as they have no immediate effect on the survival of the bacterium. A mutated 'silent' gene could become re-expressed at a future date, encoding an altered enzyme. Although this altered enzyme is likely to be inferior to the original enzyme, the possibility exists that a superior enzyme would be formed, or that a new enzyme activity would be found. In these cases, the changed gene could be advantageous to the bacterium and become established in the population.

The divergence of duplicated genes was also central to the ideas of

Horowitz (1945) and Lewis (1951). Horowitz (1945, 1965) suggested that biosynthetic pathways could have evolved by the retrograde evolution of existing enzymes. It is perhaps more difficult to see how catabolic pathways could have evolved this way. If catabolic pathways had evolved by a form of retrograde evolution, then, as Ornston (1971) remarked, homologies between an enzyme and the next enzyme of the sequence would be expected. The apparent homology between the β-ketoadipate enol-lactone hydrolase II and muconolactone isomerase (Yeh et al., 1978), two adjacent enzymes of the β-ketoadipate pathway in A. calcoaceticus, is consistent with retrograde evolution in this pathway. An alternative hypothesis involves recruitment of an existing enzyme of similar function but low activity with the new substrate. Gene duplication and enzyme improvement could then follow. As a consequence of this mechanism, Ornston (1971) suggested that homologies would exist between enzymes carrying out similar catalytic functions. Some evidence for this was provided by the work of Patel et al. (1973) in P. putida. These authors found that cis,cis-muconate lactonizing enzyme and γ-carboxy-cis,cis-muconate lactonizing enzyme, enzymes having corresponding functions in the catechol and protocatechuate branches of the β-ketoadipate pathway, were similar. Also the next enzyme of each branch, i.e. muconolactone isomerase and γ-carboxymuconolactone decarboxylase, resembled each other but not the proceeding enzymes. Thus, both retrograde evolution and enzyme recruitment may have played parts in the evolution of the β-ketoadipate pathway.

In addition to the sorts of metabolic evolution outlined above,

it is quite possible, even probable, that interspecies transfer of genetic information may be of great significance in altering the metabolic capabilities of organisms in Nature (see e.g. Wu, 1978). In this respect it may be relevant that many peripheral catabolic enzymes seem to be plasmid-coded (e.g. Williams & Worsey, 1976). This aspect, however, is beyond the scope of this Introduction since little work on 'directed laboratory evolution' has been done in this area.

In summary, experiments on 'directed evolution' have shown that when bacteria are able to gain new metabolic funtions it is by only a few mutational steps. These typically affect the rate of production of existing enzymes, the expression of previously dormant genes, the primary structure and specificity of enzymes, or the expression and specificity of transport systems.

6. Aims of this work

As we have already seen (Section 2), the enzymes for the initial attack on mandelate always show stereospecificity. Some organisms can use the L-isomer and some the D-isomer, and some both. In the last case there is either a racemase (enzyme 1 of Scheme 6) or two stereospecific dehydrogenases (enzymes 2a and 2b of Scheme 6). A. calcoaceticus NCIB8250 has an L-mandelate dehydrogenase, but cannot metabolize D-mandelate. Mutants of A. calcoaceticus NCIB8250, such as strain 41, which had evolved the capacity to oxidize this novel substrate were isolated some years ago in this laboratory (Lancaster, 1971), and some very preliminary work done with them (Fewson et al., 1976). Extracts of strain 41 slowly decoloured 2,6-dichlorophenolindophenol in the presence of D-mandelate (Lancaster, 1971). Phenylglyoxylate formation in extracts was indicated by formation of a 2,4-dinitrophenylhydrazone (Haddock, 1973) but this derivative was not identified rigorously and the experiments were only qualitative. It appeared that strain 41 could utilize D-mandelate by means of a dehydrogenase specific for D-mandelate. There was no evidence for mandelate racemization in experiments that could detect the interconversion of the two isomers in P. putida NCIB9494 (Matson, 1974). Construction by transformation of strains which could only grow on D-mandelate (Ahlquist, 1974) ruled out the involvement of L-mandelate dehydrogenase in the oxidation of D-mandelate.

Measurements of oxygen uptake indicated approximately the same activities of D- and L-mandelate dehydrogenase in whole cells, and yet maximum D-mandelate dehydrogenase activity in extracts was never

more than 20% (and usually much less) of the maximum L-mandelate dehydrogenase observed in parent strain NCIB8250 or in strain 41. A preliminary comparison of the two enzymes was made and similarities were found in (a) the location in the fraction sedimented at 100,000g for 2.5h (Lancaster, 1971), (b) inhibition of oxygen uptake by CN^-, oxalate and EDTA (Haddock, 1973). However, the new enzyme appeared less stable after heating at 37°C and storage at -60°C (Lancaster, 1971). All these conclusions were based on very low enzyme activities and many involved measurement of oxygen uptake (in which any number of factors may be rate limiting) rather than mandelate dehydrogenation itself.

The aims of the work described in this thesis were to determine if D-mandelate was metabolized by a specific D-mandelate dehydrogenase in the evolved strains and to compare the regulation of the metabolism of D-mandelate with that of L-mandelate. In addition, some comparative studies between these mutants and strain EBF65/65 were planned since this strain, in contrast to strain NCIB8250, metabolizes D- and not L-mandelate (see p.9). It was also hoped to partially characterize and compare these two enzymes metabolizing D- and L-mandelate since very little work has been done on either enzyme; indeed mandelate dehydrogenase is the least studied of all the mandelate enzymes in any bacteria. The final aim of this work would be to define the origin of the enzyme allowing the evolved strains to grow on D-mandelate.

In order to achieve these aims it was necessary to develop an assay technique for the first enzyme in D-mandelate metabolism, which turned out to be D-mandelate dehydrogenase. A method for

isolating mutants constitutive for the D-mandelate pathway enzymes was also needed since they were required for the study of regulation of the enzymes. A further advantage of having constitutive mutants was that there would be no need to use expensive inducers and so they would be useful for growing cells for a study of the enzymes themselves.

7. Mutants previously isolated from A. calcoaceticus NCIB8250

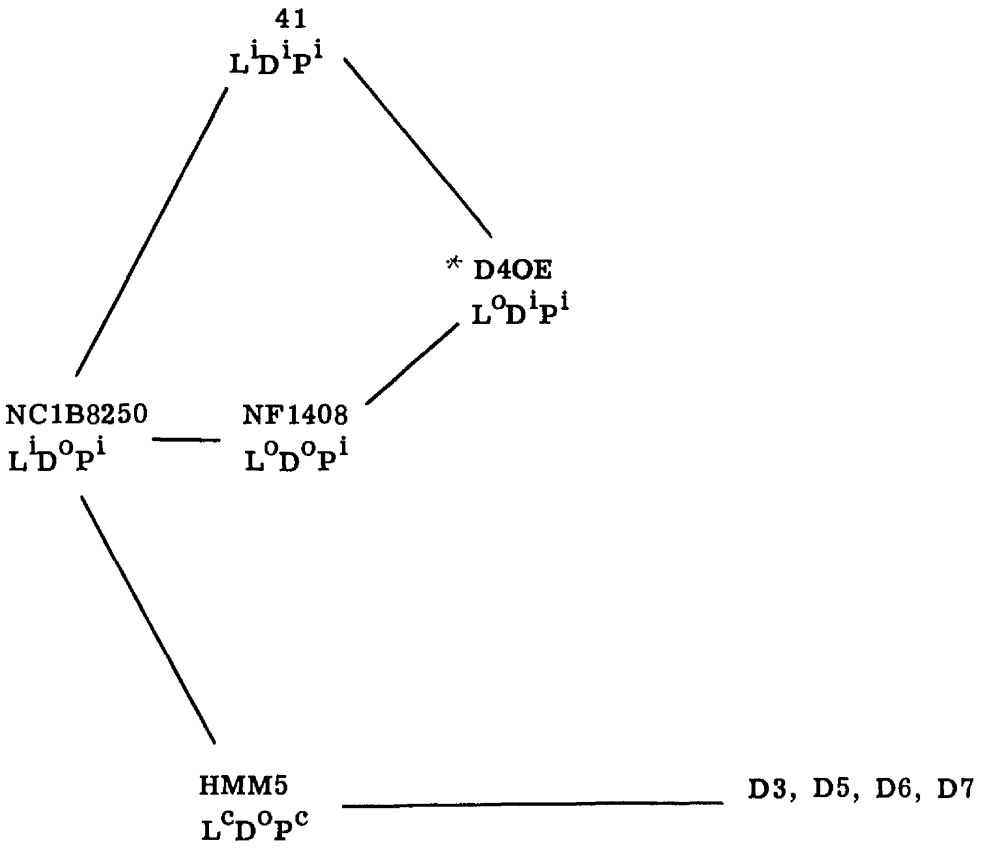
The work described in this thesis makes use of mutants isolated by previous workers (Scheme 12). Some of these mutants were used directly and some as parental strains for the isolation of constitutive mutants.

Strain NF1408, which was one of the first mutants isolated, lacks L-mandelate dehydrogenase activity but has all the rest of the enzymes of the mandelate pathway (Livingstone, 1970; Livingstone & Fewson, 1972). This mutant was isolated after treatment of the wild-type NCIB8250 with the mutagenic agent N-methyl-N'-nitro-N-nitrosoguanidine (NTG; Adelberg et al., 1965).

Both spontaneous and NTG-induced mutants able to utilize the novel carbon source, D-mandelate, were isolated by Lancaster (1971). Much of the subsequent work has been done with strain 41 (isolated in the presence of NTG), which appears typical of most mutants isolated, being very similar to strain NCIB8250, except for additional ability to use D-mandelate as source of carbon and energy for growth.

Strains able to use D-mandelate but not L-mandelate were constructed by transformation (Ahlquist, 1974; Fewson et al., 1976). DNA was isolated from strain 41 and mixed with strain NF1408 (which lacks L-mandelate dehydrogenase) on agar plates containing D-mandelate and a trace of phenylglyoxylate to serve as inducer. Most of the transformants could grow on both isomers of mandelate but about 10%, for example strains D40E and D40G, could grow only on D-mandelate and had no L-mandelate dehydrogenase activity.

Mutants constitutive for the regulon R_1 enzymes, such as HMM5, were selected on the basis of their ability to grow on phenylglyoxylate in the presence of 2-phenylpropionate, an anti-inducer of the L-



L L-mandelate dehydrogenase

D D-mandelate dehydrogenase

P phenylglyoxylate carboxy-lyase

o no enzyme activity

i inducible enzyme activity

c constitutive enzyme activity

MUTANTS PREVIOUSLY ISOLATED FROM
Acinetobacter calcoaceticus NC1B8250 (see also Table 1)

Scheme 12

*D4OE was constructed by transformation

mandelate enzymes (Fewson & Foote, 1976). Some of these mutants arose spontaneously (e.g. HMM5) and others after treatment with NTG (Moyes, 1976; Moyes & Fewson, 1976; Fewson et al., 1978). A few mutants with the ability to grow on D-mandelate were then selected from strain HMM5 (C.A. Fewson, unpublished work).

METHODS

1. Materials

All reagents were the best grade which could be obtained commercially. With the exceptions of the compounds listed below, reagents were obtained from British Drug Houses Ltd., Poole, Dorset BH12 4NN.

Bovine serum albumin (Fraction V) : Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex BN22 9AG.

Dithiothreitol (Cleland's reagent) : Boehringer Corp. Ltd., Lewes, East Sussex BN7 1LG or Calbiochem, Bishops Stortford, Herts. CM23 3AL.

D-Mandelate; L-mandelate; phenylglyoxylate : Fluka, Fluorochem Ltd., Glossop, Derbys. SK13 9NU.

Nitrilotriacetic acid : Fisons Ltd., Loughborough, Leics. LE11 ORG.

Butan 1-ol; 2,4-dinitrophenylhydrazine; toluene : Hopkin & Williams, Romford, Essex RM1 1HA.

2-Phenylpropionate : K & K, Kodak Ltd., Kirkby, Lancs. L33 7UF.

N-Methyl-N'-nitro-N-nitrosoguanidine : Koch-Light Laboratories Ltd., A. & J. Beveridge Ltd., Edinburgh EH6 5BP.

Ethyl acetate : May & Baker, Dagenham, Essex.

'Cooked meat' medium (synthetic; CM440); Andrade peptone water; peptone water; nutrient broth (CM1); nutrient agar (CM3); agar No. 1 : Oxoid Ltd., Basingstoke, Hants. RG24 OPW.

NAD : P.L. Biochemicals Inc., International Enzymes Ltd., Windsor, Berks.SL4 5NJ.

Aldehyde dehydrogenase; catalase; FAD; FMN; NAD; NADH; phenylpyruvate; thiamin pyrophosphate (cocarboxylase) : Sigma Chemical Co. Ltd., Poole, Dorset BH17 7NH.

Dulcitol : T. Kerfoot Ltd., Vale of Bardsley, Lancs.

L-Arginine monohydrochloride; L-glutamate; L-methionine : T.J. Sas & Son Ltd., London WC1B 4DF.

The purity of the mandelic acids, particularly with respect to the presence of the contaminating stereoisomer, was important in this work. The specific rotation was determined for several batches used during this work, and the melting points measured in some cases. Concentrations of mandelic acid in solution were determined from the A_{256} value after the absorption coefficient had been estimated to be 211 ± 4 (6) litre mol^{-1} cm^{-1}; this was done by carefully dissolving known weights of mandelic acid in 0.1M-HCl and then measuring the absorption spectra of the solutions of known dilution.

Mandelic acid		Optical rotation ($[\alpha]_{598}^{20}$)	Melting point (°C)
D(-); Fluka batch no.	168437 37	-154.3	133
	168437 37	-153.3	
	177298 978	-154.9	
Literature value*		-158.0	133-135
L(+); Fluka batch no.	21183 86	+155.0	135
	21183 97	+156.0	
	887798	+159.0	
Literature value*		+156.6	134-135
D,L; BDH batch no.	3013690	+0.7	122
Literature value*		0	121-122

*Mislow, 1951; Weast, 1977.

The results suggest that the optical purity of D-mandelate is not less

than 98.5%, and that of L-mandelate not less than 99.5% (the manufacturers claim $\geq$ 99%).

2. Microbiological techniques

2.1 Microorganisms:

Acinetobacter calcoaceticus strain NCIB8250 was obtained from the National Collection of Industrial Bacteria (N.C.I.B., Torry Research Station, Aberdeen AB9 8DG; Fewson,1967). A. calcoaceticus strain EBF65/65, isolated by Dr E.M. Barnes (A.R.C. Food Research Institute, Colney Lane, Norwich NR4 7UA) from a decayed carcass of frozen chicken,was obtained from Dr A. Vivian (School of Biological Sciences, Thames Polytechnic, London SE18 6PF). These two strains were the wild-types from which the various mutant strains referred to in this thesis were derived. Strains NCIB8250 and EBF65/65 are $L^{i}D^{o}P^{i}$ and $L^{o}D^{i}P^{i}$ respectively using the nomenclature:

^{o} : no enzyme activity

^{i} : inducible enzyme activity

^{c} : constitutive enzyme activity

L : L-mandelate dehydrogenase

D : D-mandelate dehydrogenase

P : phenylglyoxylate carboxy-lyase

The mutant strains listed in Table 1 are first- or second-stage mutants of strain NCIB8250 used in this work and previously isolated by other workers in the laboratory. Strains D4OE and 41 were the parents of various mutant strains (Table 2) whose isolation is described later in this thesis (Results 6.2).

A double auxotrophic mutant, C48 (ile^{-}, met^{-}), isolated from

Table 1 First- or second-stage mutants of *A. calcoaceticus* NCIB8250 isolated by previous workers

Laboratory strain number	NCIB strain number	Parent strain	Selection criterion	Enzyme complement	Isolated by	Reference
HMM5	11339	NCIB8250	constitutive synthesis of phenylglyoxylate carboxy-lyase	$L^{C}D^{O}P^{C}$	H.M. Wallace (née Moyes)	Fewson *et al.* (1978)
NF1408	10789	NCIB8250	loss of L-mandelate dehydrogenase activity	$L^{O}D^{O}P^{i}$	A. Livingstone	Livingstone (1970)
41	11209	NCIB8250	growth on D-mandelate	$L^{i}D^{i}P^{i}$	J.D. Beggs (née Lancaster)	Lancaster (1971)
D40E		NF1408 trans-formed by DNA from strain 41	growth on D-mandelate but not on L-mandelate	$L^{O}D^{i}P^{i}$	E.F. Ahlquist	Ahlquist (1974)
D3		HMM5	growth on D-mandelate	$L^{C}D^{C}P^{C}$	C.A. Fewson	(unpublished work)
D5						
D6						
D7						

Table 2 Mutants isolated from A. calcoaceticus strains D4OE and 41

Mutants of strain 41 and D4OE were selected for their ability to grow on agar plates containing phenylglyoxylate + 2-phenylpropionate (Results 6.2).

* : shown to be $L^{O}D^{C}P^{C}$

† : shown to be $L^{C}D^{C}P^{C}$

≠ : NCIB11456

§ : NCIB11457

(a) Mutants derived from D4OE

123*‡, 129*, 148*, 157*, 165*.

(b) Mutants derived from 41

213, 214, 215, 216†, 218†, 219†§, 221†,
222, 223, 224, 225†, 227, 228†, 229,
230, 234, 236†, 237, 238, 244, 247,
249, 251, 256, 259, 260, 262, 265,
267†, 271, 274, 278, 286, 288†, 289,
298, 301†, 302, 305, 308, 313†, 320,
326†, 327†, 330, 331, 332, 336, 345.

wild-type strain EBF 65/65 by Dr K. Towner (Towner & Vivian, 1976) was obtained from Dr A. Vivian. Mutants derived from strain C48 (see Results 7) are listed in Table 3.

Pseudomonas aeruginosa (NCIB8704) and Proteus vulgaris (NCIB67) were also obtained from the National Collection of Industrial Bacteria, and Escherichia coli (ATTC15224) from the American Type Culture Collection, Rockville, Maryland, U.S.A. Klebsiella aerogenes was obtained from the laboratory collection.

2.2 Characterization of the bacteria

Mutant strains were characterized by several bacteriological tests (Table 4) carried out according to the methods described by Cowan & Steel (1965). The ability to grow on the carbon compounds listed in Table 4 was used for additional characterization purposes since it is improbable that many other organisms show a similar growth pattern (Fewson, 1967a; Baumann et al., 1968). Some of the mutants differed in their ability to grow on L-mandelate, D-mandelate, or benzyl alcohol, as would be expected, but were in all other respects the same as the parental strains.

2.3 Storage of the bacteria

All strains were maintained in Oxoid cooked-meat medium (CM440) stored at 4°C. Subcultures were made into Oxoid nutrient broth (CM1) at 6-12 monthly intervals, and kept at 4°C.

3. Sterilization

3.1 Moist heat

Heat-stable solutions were autoclaved. Media containing carbon sources were autoclaved at 109°C, and all other solutions at 109°C or

Table 3 Mutants isolated from *A. calcoaceticus* strain C48

Parental strain	strain	Isolation criterion	Enzyme complement
C48	61c	growth on L-mandelate	$L^{i}D^{i}P^{i}$
C48	354 355 381	constitutive synthesis of phenylglyoxylate carboxy-lyase	$L^{o}D^{c}P^{c}$
61c	364 369 386	constitutive synthesis of phenylglyoxylate carboxy-lyase	$L^{c}D^{c}P^{c}$

Table 4 Characterization of strains of *A. calcoaceticus*

E. coli (ATCC15224) was used as a positive control in the tests for production of acid and gas from glucose, dulcitol and lactose, and a positive control in the test for methyl red and indole. *K. aerogenes* was used as a positive control for the production of acid and gas from glucose, sucrose and lactose. *Pseudomonas aeruginosa* (NCIB8704) was used as a positive control in the tests for motility, oxidase and urease. *Proteus vulgaris* (NCIB67) was used as a positive control for the urease test.

Test	NCIB8250	EBF 65/65
Shape	short rods, frequently in pairs	
Gram stain	gram-negative	
Aerobic growth	+	
Motility	-	
Colony on nutrient agar	off-white, circular, slightly raised	
Catalase	+	
Oxidase (Kovács)	-	
Urease (Christensen)	-	
Indole production	-	
Voges-Proskauer : methyl red test	-	
Voges-Proskauer : acetoin production	-	
Glucose (acid/gas)	-/-	
Sucrose (acid/gas)	-/-	
Dulcitol (acid/gas)	-/-	
Lactose (acid/gas)	-/-	
5mM-L-mandelate	+	-
5mM-D-mandelate	-	+
5mM-phenylglyoxylate	+	+
2mM-benzoate	+	+
1mM-salicylic acid	+	+
1mM-3-hydroxybenzoate	-	-
1mM-4-hydroxybenzoate	+	-
5mM-L-arginine	+	+
5mM-benzyl alcohol	+	+
2mM-benzaldehyde	+	+

120°C, for the times established by Fewson (unpublished results). The efficacy of each sterilization was verified by using a Browne's tube (Type three, A. Browne Ltd., Chancery Street, Leicester).

3.2 Dry Heat

Pipettes were wrapped in either Kraft paper or placed in metal cannisters and each sterlization (160°C; 1.75h) was checked by including a Browne's tube type one. Glass spreaders were wrapped in aluminium foil.

3.3 Filtration

The following volatile or heat-labile compounds were sterilized by filtration: benzyl alcohol, benzaldehyde, 2-phenylpropionate and thiophenoxyacetate.

Large volumes of media were sterilized by filtration through Millipore filters (GSWP04700, 0.22µm; Millipore Ltd., Abbey Road, London). For volumes of 100ml or less, Nalgene disposable sterile filter units (0.20µm; Sybron Corp., Rochester, N.Y., U.S.A.) were used. Although sold as disposable items, these filters could in some cases be used a second time following washing and resterilization (3.4). Filters were never used more than twice and recycled filters were always used for filtration of the same compound.

3.4 Ethylene Oxide

Plastic pipettes and Nalgene filter units were recycled by being washed and then sterilized by ethylene oxide. All items were sealed in polythene film and exposed to ethylene oxide (Anprolene) for 12h in a sterilizing box (AN74; H.W. Anderson Products Ltd., Clacton-on-Sea, Essex). Sterilization was verified by an anprolene exposure indicator (AN85) or by a steritest unit (AN80). All apparatus was aired for at

least 24h prior to use to remove residual gas.

4. Media

4.1 Defined media

The 'basal medium' contained 2g of KH_2PO_4 + 1g of $(NH_4)_2SO_4$ per litre, and 'basal P2 medium' contained 4g of KH_2PO_4 per litre of water, both adjusted to pH7.0 with NaOH.

In the preparation of sterile growth media, heat-stable carbon and carbon + nitrogen sources were dissolved in the appropriate basal media, which were then adjusted to pH7.0 with NaOH, made up to a correct final volume with distilled water, and autoclaved. Volatile and heat-labile carbon sources were adjusted to pH7.0, sterilized by filtration and then added aseptically to the sterile basal medium.

Immediately before inoculation, sterile 2% (w/v) $MgSO_4.7H_2O$ (20ml per litre) was added to sterile media containing carbon sources.

When the auxotroph C48, or any mutants of this strain, were grown the growth medium was supplemented with D,L-isoleucine and L-methionine (final concn. 0.05g per litre of each). Suitable concentrations of the amino acids were dissolved in 2% (w/v) $MgSO_4.7H_2O$ before sterilization.

In this thesis the term 'salts medium' refers to basal medium + 20ml 2% (w/v) $MgSO_4.7H_2O$ per litre; 'salts P2 medium' refers to basal P2 medium + 20ml 2% (w/v) $MgSO_4$; 5mM-L-glutamate/salts medium refers to 5mmol-L-glutamate per litre of salts medium.

4.2 Nutrient broth

Nutrient broth (Oxoid CM1; $13gl^{-1}$) was dispensed in 5ml or 10ml

volumes in tablet and universal bottles respectively for storage of cultures, or in 50ml or 100ml volumes in 250 and 500ml conical flasks respectively for growing inocula.

4.3 Agar plates

4.3.1 Nutrient agar plates

The agar (Oxoid CM3; $28gl^{-1}$) was boiled and stirred to dissolve it and then autoclaved. The sterile molten medium was cooled to $55^{o}C$ in a thermostatically controlled water bath, and then poured into Petri dishes (9cm diameter: Sterilin Ltd., Teddington, Middlesex). Plates were left inverted for 24h at $37^{o}C$ to dry.

4.3.2 Agar plates containing defined media

Oxoid agar No. 1 [L11; 1.5% (w/v)] was mixed with basal medium containing the appropriate carbon source and then autoclaved. If the carbon source was heat-labile it was sterilized by filtration and added to the sterile, molten agar/basal medium at $55^{o}C$. Before pouring into plates, 20ml 2% (w/v) sterile $MgSO_4.7H_2O$ was added to each litre of medium. The plates were dried at $37^{o}C$ for 24h.

5. Turbidity measurement

Turbidities of bacterial suspensions were estimated by measuring the absorbance relative to air at 500nm in 1cm light-path cuvettes using a Unicam SP800 Spectrophotometer (Pye Unicam Instruments Ltd., 57b, West Harbour Rd., Granton, Edinburgh EH5 1PP) connected to a Servoscribe Chart Recorder (Smith's Industries Ltd., Kelvin House, Wembley, Middlesex). Up to four cuvettes were read in the automatic sample changer of the spectrophotometer. At high bacterial concentrations (greater than $A_{500} = 0.6$), turbidity measurements were corrected for the non-linear relationship between observed A_{500} and actual turbidity by using a standard curve.

6. Growth of bacteria

6.1 Growth of cultures

Culture volumes of 50ml and 80ml were grown in 250ml conical flasks stoppered with polystyrene foam bungs (A. & J. Beveridge Ltd., 5, Bonnington Road Lane, Edinburgh EH6 5BP). The cultures were usually grown on a rotary shaker (Mk. V. Orbital Shaker, L.H. Engineering Co. Ltd., Stoke Poges) moving at about 180 oscillations min^{-1}, in rooms thermostatically maintained at 23°C or 30°C as appropriate.

Cultures were grown in 400 and 500ml quantities of medium in 1 litre flat-bottomed flasks fitted with side-arms to facilitate sampling (Harvey et al., 1968). The flask necks were closed with silicone rubber bungs each fitted with a glass tube plugged with non-absorbent cotton wool, and the side-arms were covered by Morton Culture Tube Closures with fingers (T1390-20; Scientific Products, American Hospital Supply Corp., Evanston, Illinois, U.S.A.). The cultures were maintained at 23°C or 30°C in a water bath whose temperature was controlled with a Circotherm unit (Shandon Scientific Co. Ltd., Cromwell Place, London). The cultures were grown under conditions of vigorous aeration on the apparatus described by Harvey et al. (1968). This apparatus consists of magnetic drive assemblies which effect the high speed rotation of magnetic stirring bars (45mm, polypropylene coated; stirring at approximately 300 rpm). Air (200ml min^{-1}) was passed through the glass tubes in the silicone bungs; these glass tubes did not enter the culture.

Cultures of 3 litre volumes were grown in 10 litre, flat-bottomed flasks each having a plug of non-absorbant cotton wool through which passed a glass pipette (10ml). The cultures were grown at 30°C under conditions of vigorous aeration produced by a 45mm polypropylene-coated

magnetic bar stirring at approximately 300 rpm on the apparatus described by Harvey et al. (1968). Air (200ml min^{-1}) was passed through the glass pipette over the culture.

6.2 Nutrient broth inocula

Inocula used for growing the bacteria in defined media were prepared immediately before use. Conical flasks (250 or 500ml) containing 50ml or 100ml nutrient broth were inoculated (0.1%, v/v) with the nutrient broth stock culture and grown without shaking. These cultures were grown at either 23°C for 48h or 72h and used as inocula for growing bacteria in defined media at 23°C, or at 30°C for 24h or 48h when used as inocula for growing bacteria in defined media at 30°C.

6.3 Growth of strains EBF65/65, NCIB8250, and mutants derived from NCIB8250, on the carbon compounds included as part of the characterization experiment

Nutrient broth cultures of the strains tested were grown for 24h at 30°C. The bacteria were inoculated (0.2%, v/v) into 50ml of sterile salts media containing the appropriate carbon source. The cultures were grown at 30°C on the rotary shaker. Growth was estimated as 'growth' or 'no growth' 16h, 24h, 40h and 48h after inoculation. Results in Table 4 refer to growth after 48h.

6.4 Growth of mutants isolated from strain 41, able to grow well on benzyl alcohol, for measurement of phenylglyoxylate carboxy-lyase activity

Nutrient broth cultures of the mutants derived from strain 41 were grown for 24h at 30°C. These bacteria were inoculated (8%, v/v) into 50ml sterile salts medium containing the appropriate carbon source, grown at 30°C with shaking and harvested when the turbidity reached A_{500} = approximately 0.4.

6.5 Growth of mutants HMM5, 148, 219 and D6 in 5mM-L-glutamate/salts P2 medium to determine the effect of growth temperature on D- and L-mandelate dehydrogenase activity

Nutrient broth cultures of mutants HMM5, 148, 219 and D6 were grown at 30°C for 24h. These bacteria were inoculated (25%, v/v) into appropriate medium so that the final composition was 5mM-L-glutamate/salts P2 (in 400ml). The cultures were grown at 23°C or 30°C and harvested when the turbidity reached A_{500} = 0.6-0.8.

6.6 Growth of mutant strains derived from strains D40E, 41 and HMM5 in 5mM-L-glutamate/salts P2 medium for the measurement of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase activities

Nutrient broth cultures of the mutants derived from strains D40E, 41 and HMM5 were grown for 24h at 23°C. These bacteria were inoculated (25%, v/v) into flasks of appropriate medium so that the final composition was 5mM-L-glutamate/salts P2 (in 80ml). The cultures were grown at 23°C and harvested when the turbidity reached A_{500} = 0.4-0.6 (i.e. 2-7h after inoculation).

6.7 Growth of mutant 148 in 5mM-D-mandelate + 5mM-L-glutamate/salts medium for development of the D-mandelate dehydrogenase assay

A nutrient broth culture of mutant 148 was grown for 24h at 30°C. These bacteria were inoculated (0.25%, v/v) into 5mM-D-mandelate + 5mM-L-glutamate/salts medium (500ml) and grown at 30°C and harvested when the turbidity reached A_{500} = 0.4-0.5.

6.8 Growth of cultures in 5mM-L-glutamate/salts P2 medium with and without additional inducers, repressors or anti-inducer

Nutrient broth cultures were grown at 23°C for 48h or 72h. These bacteria were inoculated (25%, v/v) into flasks of appropriate medium so that the final composition was 5mM-L-glutamate/salts P2 (in 400ml) or into the same medium containing the following compounds: 5mM-L-mandelate; 5mM-D-mandelate; 5mM-phenylglyoxylate; 5mM-phenylglyoxylate + 2.5mM-2-phenylpropionate; 2mM-thiophenoxyacetate; 5mM-phenylglyoxylate + 5mM-succinate. The cultures were grown at 23°C and harvested when the turbidity reached A_{500} = 0.5-0.7 (i.e. 3-5h after inoculation).

6.9 Growth of mutant HMM5 in 5mM-L-glutamate/salts P2 medium at 30°C

A nutrient broth culture of mutant HMM5 was grown at 30°C for 24h. These bacteria were inoculated (25%, v/v) into medium so that the final composition was 5mM-L-glutamate/salts P2 (in 400ml). The cultures were grown at 30°C and harvested when the turbidity reached A_{500} = 0.6-0.7 (i.e. 2-3h after inoculation).

6.10 Growth of mutant HMM5 in 40mM-L-glutamate/salts P2 medium

A nutrient broth culture of mutant HMM5 was grown for 24h at 30°C. These bacteria were inoculated (0.6%, v/v) into 40mM-L-glutamate/basal P2 medium containing 120ml of 2% (w/v) $MgSO_4$ + 2ml of a mixture of trace metals prepared as follows (Beggs & Fewson, 1977). Nitrilotriacetic acid (50g, 'Chel NTA') was dissolved in 1M-NaOH (625ml) and the solution adjusted to pH7.0 with 5M-HCl. Salts were then added in the following order, each being allowed to dissolve before the next was added: $FeSO_4.7H_2O$, 1.1g; $Na_2MoO_4.2H_2O$, 50mg;

$MnSO_4.4H_2O$, 50mg; $ZnSO_4.7H_2O$, 50mg; $CuSO_4.5H_2O$, 25mg; $CoCl_2.6H_2O$, 25mg. The solution was made up to 1 litre and autoclaved at 109°C.

The bacteria were grown at 30°C and harvested when the turbidity reached A_{500} = 2.7 (i.e. approx. 20h after inoculation).

6.11 Growth of the wild-type strain EBF 65/65, and mutants derived from this strain

Nutrient broth cultures of these strains were grown for 40h at 23°C. These bacteria were inoculated (25%, v/v) into medium so that the final composition (in 400ml) was 5mM-L-glutamate/salts P2 or 5mM-L-glutamate + 5mM-phenylglyoxylate/salts P2 supplemented with D,L-isoleucine + L-methionine. The cultures were grown at 23°C and harvested when the turbidity reached A_{500} = 0.5-0.7 (i.e. 2-3h after inoculation).

7. Harvesting and storage of bacteria

7.1 Routine method

Cultures were harvested by centrifugation in 750ml polypropylene bottles (maximum volume 500ml; 69650, MSE Ltd., Buckingham Gate, London) at 6,000g for 20 min at 4°C in the MSE Mistral 6L centrifuge. Each pellet was resuspended in about 20ml ice-cold, sterile basal medium and recentrifuged in 50ml polypropylene centrifuge tubes (59407; MSE) at 12,000g for 30 min at 4°C in the MSE Highspeed 18 centrifuge. The supernate was carefully decanted and the bacterial pellets were stored in these tubes covered with parafilm (Fisons Ltd., Loughborough, Leics. LE11 ORG) at -18°C until required.

7.2 Harvesting and storage of strain 41, wild-type strain EBF65/65 and mutants derived from these strain for measurement of phenylglyoxylate carboxy-lyase activity

A 25ml portion of culture was harvested by centrifugation in 50ml polypropylene centrifuge tubes at 12,000g for 30min at 4°C in the MSE Highspeed 18 centrifuge. The bacterial pellet was stored at -18°C in these tubes covered with parafilm until required for the assay of phenylglyoxylate carboxy-lyase.

8. Isolation of mutants

8.1 Isolation and identification of mutants constitutive for phenylglyoxylate carboxy-lyase

Constitutive mutants were isolated by plating the parent strain on medium containing anti-inducer (Fewson et al., 1978). Portions (0.1ml) of a 24h nutrient broth culture were spread on agar plates containing 2mM-2-phenylpropionate + 2mM-phenylglyoxylate/salts medium. A few (1-5) crystals of NTG were placed on the centre of some of the plates. Presumptive mutant colonies appearing during 10d incubation at 30°C were picked off into nutrient broth, cloned on 2-phenylpropionate + phenylglyoxylate medium and then tested for constitutive synthesis of phenylglyoxylate carboxy-lyase.

Mutants which constitutively synthesize phenylglyoxylate carboxy-lyase formed much smaller colonies on benzyl alcohol agar plates (described in Results 6.2; Fig. 18), than mutants with an inducible enzyme. Colonies appearing on nutrient agar plates were all approximately the same size, whether constitutive or not.

Possible mutants and control strains were grown in nutrient broth (50ml) for 24h at 30°C. Serial dilutions (1 in 10 and 1 in 100) were

made in basal medium, and one drop of the 10^{-4} and 10^{-5} dilutions placed on nutrient agar plates or agar plates containing 5mM-benzyl alcohol/salts medium. The plates were incubated at 30°C and the size of colonies appearing after 24h and 48h noted.

Mutants known to synthesize phenylglyoxylate carboxy-lyase constitutively, and those with an inducible enzyme were always included as controls. Confirmation of constitutive synthesis of phenylglyoxylate carboxy-lyase was established by measuring the enzyme activity after growth on the non-inducing carbon source, L-glutamate (for examples see Table 18).

8.2 Isolation of mutants of NCIB8250 and NF1408 able to grow on D- or L-mandelate

Table 5 summarizes the conditions used in this thesis and by J.D. Beggs (Lancaster, 1971) and C.A. Fewson (unpublished work) to enrich mutants of strain NCIB8250 and NF1408. The methods used in this thesis will now be described in detail.

Nutrient broth cultures of strains NCIB8250 and NF1408 were grown at 30°C for 24h or 23°C for 48h. Up to 40 flasks each containing salts medium (50ml) containing one of the following carbon sources: 5mM-D-mandelate; 5mM-D-mandelate + 0.25mM-phenylglyoxylate; 5mM-L-mandelate; 5mM-L-mandelate + 0.25mM-phenylglyoxylate, were inoculated with either 4ml, 2ml, 1ml or 0.5ml portions of nutrient broth per flask, each inoculum size being used for up to 10 flasks. The cultures were shaken at 30° or 23°C and the number of flasks showing growth was monitored over 5d. Samples were taken and cloned.

Table 5 Summary of the conditions used in this work and elsewhere to enrich mutants of strain NCIB8250 and NF1408 able to grow on D- or L-mandelate

Various volumes of nutrient broth cultures grown for 24h at 30°C or 48h at 23°C were used to inoculate 5mM-D-mandelate or 5mM-L-mandelate/salts medium (in 50ml) with or without 0.25mM-phenylglyoxylate. The cultures were incubated at 23°C or 30°C on a rotary shaker, and the number of flasks showing growth monitored over 5d.

D + P : 5mM-D-mandelate + 0.25mM-phenylglyoxylate/salts

D : 5mM-D-mandelate/salts

L + P : 5mM-L-mandelate + 0.25mM-phenylglyoxylate/salts

L : 5mM-L-mandelate/salts

J.D.B. : J.D. Beggs (Lancaster, 1971)

C.A.F. : C.A. Fewson (unpublished work)

C.A.H. : C.A. Hills (this thesis)

Starting strain	Enrichment media	Total number of flasks inoculated	Number of flasks inoculated — Volume of inoculum (ml) 4	2	1	0.8	0.5	0.4	0.2	0.1	Temp. of growth (°C)	Isolated by
8250	D + P	32			32						30	C.A.F.
		8				2		2	2	2	30	J.D.B.
		40	10	10	10		10				30	C.A.H.
		39	10	10	10		9				30	C.A.H.
8250	D	40	10	10	10		10				30	C.A.H.
NF1408	D + P	32			32						30	C.A.F.
		39	10	10	10		9				30	C.A.H.
		39	10	10	10		9				23	C.A.H.
NF1408	L + P	32			32						30	C.A.F.
		39	10	10	10		9				30	C.A.H.
		39	10	10	9		10				30	C.A.H.
NF1408	L	38	10	10	9		9				30	C.A.H.

8.3 Isolation of mutants of strain C48 able to grow on L-mandelate

Nutrient broth cultures of strain C48 were grown for 24h at 30°C. Nineteen flasks containing 5mM-L-mandelate/salts medium (50ml) supplemented with D,L-isoleucine and L-methionine (0.05g per litre of each) were inoculated with either 4ml, 2ml, 1ml or 0.5ml portions of nutrient broth per flask. The 4ml, 2ml or 1ml inoculum size were used for 5 flasks, and 4 flasks were inoculated with 0.5ml of nutrient broth per flask. The flasks were incubated at 30°C with shaking and the number showing growth monitored over 5d. Samples were taken and cloned.

9. Disruption of bacteria

Bacterial suspensions were disrupted with Dawe soniprobes (Type 1130A; Dawe Instruments Ltd., London). Two soniprobes were used in the work described in this thesis, number 1 and number 3. For the majority of the work, and unless stated otherwise, probe number 3 was used at setting 6. Probe number 1, setting 6, gave similar results to number 3 until it was completely overhauled and the transducer replaced. It then became less efficient and enzyme specific activities measured in the extracts were low (see Table 10a and b in comparison to Tables 9 and 11).

9.1 Preparation of extract

9.1.1 Preparation of extract for measurement of D- and L-mandelate dehydrogenase, benzaldehyde dehydrogenase, catechol 1, 2-oxygenase, and NADH oxidase

Bacteria were resuspended in 0.1M-potassium phosphate buffer, pH7.5 to A_{500} = 5. Five ml was placed in a chilled 2 dram vial

and positioned in a chilled brass holder (Holms & Bennett, 1971). This was screwed onto the horn of a 13mm probe of the Dawe soniprobe and lowered into an ice-water slurry. Disruption was effected at a current of 3A for seven 30s periods alternated with 'cooling' periods of 30s. The homogenate was centrifuged at 12,000g for 30min at 4°C in the MSE Highspeed 18 centrifuge to remove whole bacteria and debris. The supernate ('extract') was kept on ice until required for assays of enzyme activity and protein concentration.

9.1.2 Preparation of extract of mutant 219 for comparison of D- and L-mandelate dehydrogenases

Bacteria were resuspended to A_{500} = 34 in 0.1M-potassium phosphate buffer, pH7.5. The bacterial suspension (70ml) was placed in a Rosett (1965) cell surrounded by an ice-water slurry. The 1cm tip of the Dawe soniprobe (number 3) was lowered into the suspension. Disruption was effected at a current of 3A for fourteen 30s periods alternated with 'cooling' periods for 30s. The homogenate was centrifuged at 12,000g for 30min at 4°C in the MSE Highspeed 18 centrifuge to remove whole bacteria and debris. The supernate was stored at -18°C in small samples (1 or 2ml), and thawed and diluted in 0.1M-potassium phosphate buffer, pH7.5 as required.

9.2 Preparation of a membrane fraction of mutant 219 to identify the reaction product from the oxidation of D-mandelate and to measure oxygen uptake

Bacteria were resuspended to high turbidity (A_{500} = 17 to 62) in 0.1M-potassium phosphate buffer, pH7.5. The bacterial suspensions (24 to 84ml) were placed in suitable Rosett (1965) cells, surrounded by an ice-water slurry. The 1cm tip of the Dawe soniprobe (number 3)

was lowered into the suspension. Disruption was effected at a current of 3A for fourteen 30s periods alternated with "cooling" periods of 30s. The homogenate was centrifuged at 2,500g for 10min at 4°C in the MSE Highspeed 18 centrifuge to remove whole cells. The supernate was then centrifuged at 100,000g for 2.5h at 4°C in the Beckman L2-65B Ultracentrifuge (Beckman Instruments Ltd., Queensway, Glenrothes, Fife) in a Type 65 rotor. The supernate was carefully removed and the pellet was resuspended in 0.1M-potassium phosphate buffer (6-9ml) and stored at -18°C ('membrane fraction').

10. Treating bacteria with toluene

Bacteria were treated with toluene by the method of Beggs & Fewson (1977). For the determination of phenylglyoxylate carboxy-lyase (benzoyl-formate decarboxylase; EC 4.1.1.7), bacteria were resuspended in ice-cold basal medium to a known A_{500} of about 1. A portion of the suspension (2ml) was added to 10µl toluene in 6 x 0.75 inch test tubes and mixed for 30s on a vortex mixer. The tube was covered with an Oxoid metal test tube cap and incubated at 27°C for 4h.

For the determination of benzyl alcohol dehydrogenase bacteria were resuspended to a known A_{500} of about 1 in ice-cold salts medium containing 2mM-dithiothreitol. A portion of the suspension (2ml) was added to 0.1ml 4% (v/v) toluene in ethanol in a 6 x 0.75 inch test tube and mixed for 30s. The tube was covered with a metal cap and incubated at 27°C for 4h.

11. Enzyme assays

Enzymes were assayed in quartz, glass or plastic 1cm light-path

cuvettes in a Unicam SP800 spectrophotometer connected to a Servoscribe chart recorder. The scale on the chart recorder could be expanded up to ten times in order to measure small absorbance changes. The temperature of an external cuvette holder and of the spectrophotometer cuvette carriage was maintained at 27°C by a Circotherm unit which heated and circulated water from an adjacent water bath. The assay buffer and distilled water were kept in the water bath. All the reaction components, except the substrate, were added to the cuvettes which were standing in the external cuvette holder. Micropipettes (Eppendorf Marburg Mikropipet, V.A. Howe & Co. Ltd., London W11) and occasionally Hamilton syringes (Magnus Scientific, Sandbach, Cheshire) were used to dispense volumes less than 500µl. These components of the reaction mixture were mixed by inversion after covering the cuvettes with parafilm; the cuvettes were then placed in the cuvette carriage. The reactions were initiated by addition of substrate and mixing with 'plumpers' (Calbiochem, Los Angeles, California, U.S.A.).

Up to four cuvettes could be placed in the spectrophotometer and the absorbance measured sequentially at 10-15s intervals for up to 5min by means of a programmed cuvette changer.

Enzyme units are defined as µmol substrate converted min^{-1}; specific activities are given as munits of enzyme (mg protein)$^{-1}$.

11.1 D-Mandelate dehydrogenase

The development of this routine assay system is described in the Results Section. D-Mandelate dehydrogenase (DMDH) was assayed in extracts prepared from ultrasonically treated bacteria (Methods 9.1.1). The reaction mixture contained (in a total volume of 3.0ml):

200µmol KH_2PO_4-K_2HPO_4 buffer, pH7.5,

200nmol DCIP

1µmol PMS

10mg BSA (dissolved in 0.2ml 0.1M-potassium phosphate buffer, pH7.5)

extract (usually 100µl)

1.5µmol D-mandelate (adjusted to pH7.5) to initiate the reaction

The rate of reduction of the dye to its colourless form was followed at 600nm. The oxidation of 1µmol D-mandelate/assay gives $\Delta A_{600} = -6.87$.

11.2 L-Mandelate dehydrogenase

L-Mandelate dehydrogenase (LMDH) was measured either by the method of Livingstone & Fewson (1972) or by a modification of this method which involved adding PMS and BSA to the assay, in other words a system identical with that used to measure D-mandelate dehydrogenase.

11.2.1 Original L-mandelate dehydrogenase

L-Mandelate dehydrogenase was assayed in extracts prepared from ultrasonically treated bacteria (see Methods 9.1.1). The reaction mixture contained (total volume of 3.0ml):

200µmol KH_2PO_4-K_2PO_4 buffer, pH7.0

200nmol DCIP

extract (usually 100µl)

1.5µmol L-mandelate (adjusted to pH7.0) to initiate the reaction

The rate of reduction of the dye to its colourless form was measured at 600nm. The oxidation of 1µmol L-mandelate/assay gives a $\Delta A_{600} = -6.87$.

11.2.2 Modified L-mandelate dehydrogenase assay

L-Mandelate dehydrogenase was assayed in extracts prepared from ultrasonically treated bacteria (Methods 9.1.1). The reaction mixture contained (in a total volume of 3.0ml):

200µmol KH_2PO_4-K_2HPO_4 buffer, pH7.0

200nmol DCIP

1µmol PMS

10mg BSA (dissolved in 0.2ml 0.1M-potassium phosphate buffer, pH7.5)

extract (usually 100µl)

1.5µmol L-mandelate (adjusted to pH7.0) to initiate the reaction

The rate of reduction of dye was followed at 600nm. The oxidation of 1µmol L-mandelate/assay gives $\Delta A_{600} = -6.87$.

Generally for both D-mandelate dehydrogenase (11.1) and L-mandelate dehydrogenase (11.2.1 and 11.2.2) triplicate determinations were made and corrected for endogenous activity by subtracting the value determined in a fourth reaction mixture which lacked substrate.

11.3 Phenylglyoxylate carboxy-lyase

Phenylglyoxylate carboxy-lyase (PC) was measured as described by Beggs & Fewson (1977). After treatment with toluene (Methods 10) the samples were transferred to 4 x 0.4 inch test tubes to facilitate sampling and assayed in reaction mixtures containing (total volume 3.0ml):

200µmol KH_2PO_4-K_2HPO_4 buffer, pH7.0

6µmol NAD^+

200nmol thiamin pyrophosphate (dissolved in 0.1ml 0.25M-KH_2PO_4-K_2HPO_4 buffer, pH7.0)

1 unit aldehyde dehydrogenase (added in 0.1ml of a solution in 1mM-dithiothreitol)

toluenised bacterial suspension (usually 100µl)

15µmol freshly prepared phenylglyoxylate (adjusted to pH7.0) to initiate the reaction

The rate of NAD^+ reduction was followed at 340nm. The decarboxylation of 1µmol phenylglyoxylate to benzaldehyde, and the oxidation of benzaldehyde by the excess aldehyde dehydrogenase gives a $\Delta A_{340} = +2.07$.

11.4 Benzaldehyde dehydrogenase

Benzaldehyde dehydrogenase (BDH) was measured as described by Beggs & Fewson (1977) in extracts prepared from ultrasonically treated bacteria (Methods 9.1.1). The reaction mixtures contained (in a total volume of 3.0ml):

200µmol $Na_4P_2O_7.10H_2O$ buffer, pH9.0

6µmol NAD^+

50µmol KCl

extract (usually 100µl)

300nmol freshly prepared benzaldehyde to initiate the reaction

The rate of reduction of NAD^+ was followed at 340nm. The oxidation of 1µmol benzaldehyde/assay gives $\Delta A_{340} = +2.07$.

11.5 Benzyl alcohol dehydrogenase

Benzyl alcohol dehydrogenase (BADH) was measured as described by Beggs et al. (1976). After treatment with toluene and ethanol (Methods 10), the samples were transferred to 4 x 0.4 inch test tubes to facilitate sampling and assayed in reaction mixtures containing (total volume 3.0ml):

200µmol $Na_4P_2O_7.10H_2O$. buffer, pH9.0

6µmol NAD^+

toluenised bacterial suspension (usually 100µl)

600nmol freshly prepared benzyl alcohol to initiate the reaction

The rate of reduction of NAD^+ was followed at 340nm. The oxidation of 1µmol benzyl alcohol/assay gives $\Delta A_{340} = +2.07$.

11.6 NADH oxidase

NADH oxidase was measured by the method of Livingstone (1970) in extracts prepared from ultrasonically treated bacteria (Methods 9.1.1). The reaction mixture contained (in a total volume of 3.0ml):

200µmol KH_2PO_4-K_2HPO_4 buffer, pH7.0

extract (usually 100µl)

200µg NADH (dissolved in 0.1M-potassium phosphate buffer, pH7.0) to initiate the reaction

The rate of oxidation of NADH was followed at 340nm. The oxidation of 1µmol NADH/assay gives $\Delta A_{340} = -2.07$.

11.7 Catechol 1,2-oxygenase

Catechol 1,2-oxygenase (CO; E.C.1.13.1.1.) was assayed by the method of Hegeman (1966a) in extract prepared from ultrasonically treated bacteria (Methods 9.1.1). The reaction mixture contained (in a total volume of 3.0ml):

200µmol KH_2PO_4-K_2PO_4 buffer, pH7.0

4µmol EDTA (adjusted to pH7.0)

extract (usually 100µl)

300nmol freshly prepared catechol to initiate the reaction

The rate of appearance of cis, cis-muconate was followed at 260nm. The production of 1µmol cis, cis-muconate/assay gives $\Delta A_{260} = +5.6$.

12. Identification of mandelate pathway intermediates

12.1 Phenylglyoxylate in the presence of D-mandelate

Phenylglyoxylate concentration was determined as described by Beggs (1974). A sample (1ml) was added to 3ml of ice-cold 13.4% (w/v) perchloric acid, left on ice for 10min and centrifuged at 12,000g for 10min. The supernate (3.5ml) was extracted with 5ml chloroform by vortex mixing for 15s. The aqueous phase was removed by suction and 3ml of the chloroform phase was extracted with 5ml 0.2M-NaOH by vortex mixing for 15s. The aqueous phase (3ml) was acidified by addition of 1ml M-HCl. The absorbance was then measured at 253nm against a reference solution of M-HCl in 1cm light-path quartz cuvettes using a Unicam SP800 spectrophotometer. Phenyl-glyoxylate concentrations were calculated by reference to a standard curve (e.g. Fig. 1). D-Mandelate did not interfere with the assay for phenylglyoxylate (Fig. 1).

12.2 Benzaldehyde

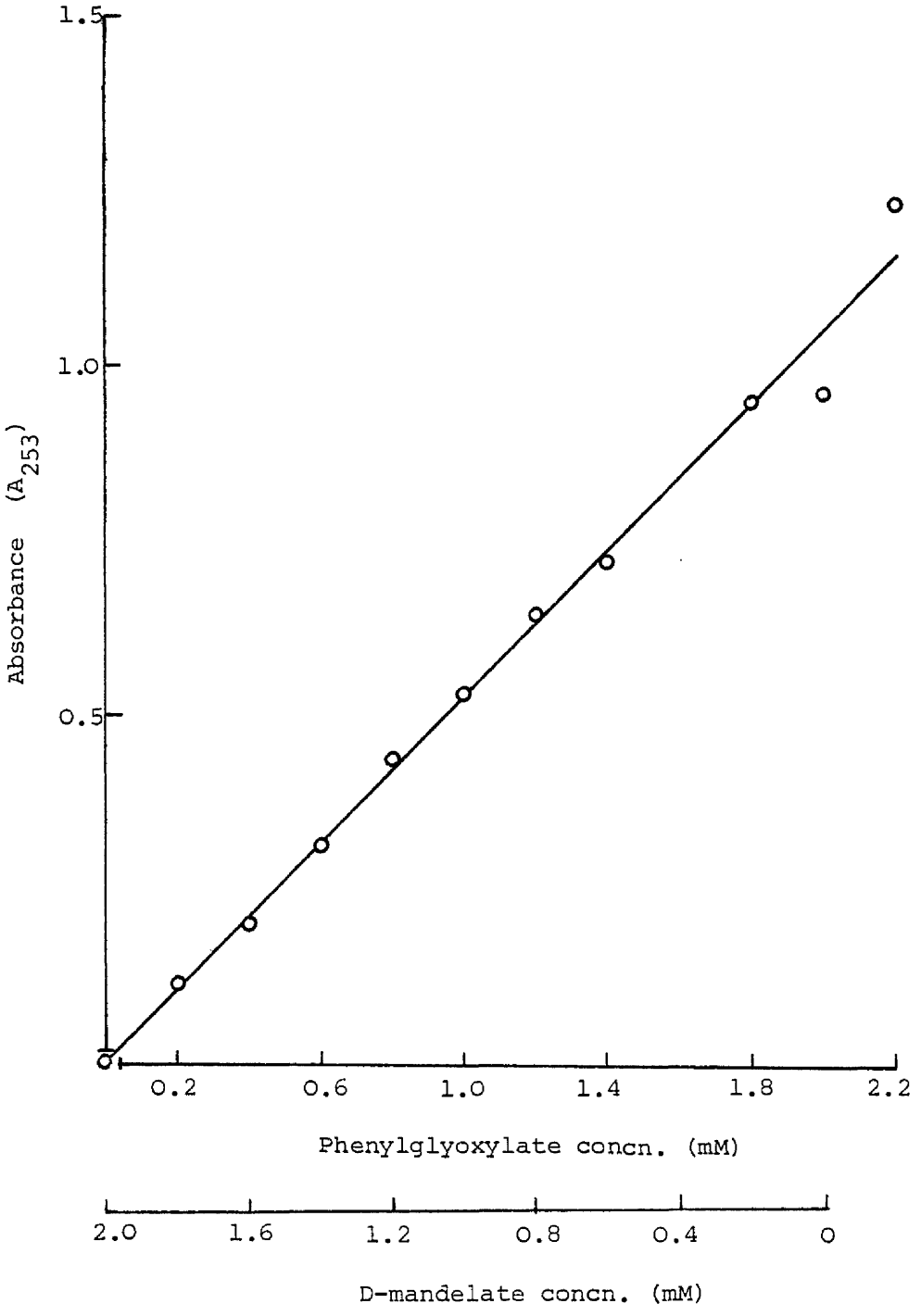
Benzaldehyde was identified by the method of Cook *et al.* (1975). A sample (1ml) was added to 0.1ml M-NaOH + 10ml *n*-hexane (product no. 28488; this preparation has low u.v. absorption) and mixed for 30s. The absorption spectrum of the top hexane layer was measured against a hexane reference in 1cm light-path quartz cuvettes using a Unicam SP800 spectrophotometer, and compared to that of an authentic sample treated in a similar manner.

13. Thin layer chromatography

Unknowns and standards were applied as solutions in diethyl-ether to Serva Feinbiochemica Silufol sheets, UV 254 (Micro-Bio Laboratories, London). The solvent system was butan 1-ol saturated

Fig. 1 Standard curve for the estimation of phenylglyoxylate in the presence of D-mandelate

Mixtures of phenylglyoxylate and D-mandelate solutions were treated with perchloric acid, extracted first into chloroform, then into NaOH, and finally acidified with HCl before measuring the absorbance at 253nm (Methods 12.1).


1.5
1.0
0.5
Absorbance (A_{253})
0.2
0.6
1.0
1.4
1.8
2.2
Phenylglyoxylate concn. (mM)
2.0
1.6
1.2
0.8
0.4
0
D-mandelate concn. (mM)

with ammonium carbonate buffer, pH9.7 (Fewster & Hall, 1951), freshly prepared for each experiment. The ammonium carbonate buffer contained 78.5g $(NH_4)_2CO_3$ + 75ml ammonia (sp.gr. 0.88) per litre. The chromatograms were developed by ascending chromatography at room temperature (approx. 17°C) and the spots located under ultraviolet light.

14. Preparation and chromatography of 2,4-dinitrophenylhydrazones

Sample (5ml) was added to 7.5ml of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2M-HCl, vortex mixed for 30s, and left at room temperature for 20 min. This mixture was extracted using 5ml ethyl acetate with vortex mixing for 30s. After discarding the aqueous phase, the organic phase was extracted by mixing with 3ml 10% (w/v) Na_2CO_3 for 30s. The organic layer was discarded and the absorption spectrum of the aqueous layer measured against a reference solution of 10% Na_2CO_3 in 1cm light-path quartz cuvettes using a Unicam SP8-100 spectrophotometer. The aqueous phase was acidified by adding 5M-HCl until effervescence ceased and extracted a number of times with ethyl acetate. The pooled organic phases from these extractions were concentrated by evaporation for chromatography. The 2,4-dinitrophenylhydrazones were separated by ascending chromatography on Whatman No. 1 paper. The solvent system was butan 1-ol/ethanol/ammonium carbonate buffer (40:11:19 by vol), freshly prepared for each experiment. Ammonium carbonate buffer contained 7.2g $(NH_4)_2CO_3$ + 5.2ml ammonia (sp.gr. 0.88) per litre. The chromatograms were developed at room temperature (approx. 17°C).

15. Measurements of oxygen uptake

Two oxygen electrodes (Rank Brothers,Bottisham, Cambridge CB5 9DA) were maintained at 27°C by a Circotherm unit which circulated water from an adjacent water bath through the electrode jackets. The electrodes were connected to a Servoscribe chart recorder, and the procedure of Robinson & Cooper (1970) was used to calibrate the apparatus; in this method oxygen is consumed in the stoicheiometric oxidation of a known (determined spectrophotometrically) amount of NADH. The NADH is oxidised by PMS, which in turn is oxidised by molecular oxygen. The H_2O_2 formed is then broken down by catalase and the net reaction becomes:

$$NADH + H^+ + \tfrac{1}{2}O_2 \longrightarrow NAD^+ + H_2O$$

The assay buffer and distilled water were maintained at 27°C in the water-bath, and were air-saturated by vigorously stirring the solutions with magnetic stirring bars and magnetic drive assemblies using the apparatus described by Harvey et al. (1968). The potassium phosphate buffer (2ml) pH7.0 or 7.5, distilled water and bacterial preparation (and BSA, PMS and DCIP if added at this stage) were pipetted into each incubation vessel to a total volume of 3ml. The perspex disc was then carefully screwed down into position so that no air bubbles were trapped. The endogenous rate of oxygen uptake was recorded for at least 5min. All further additions were made through the small hole in the perspex disc using Hamilton syringes and the rate of oxygen consumption measured. L- and D-mandelate were added as 100µl of 0.15M solutions adjusted to pH7.0 and 7.5 respectively. DCIP was added as 100µl of a 2mM solution, PMS as 100µl of a 10mM solution, and BSA as 200µl of a 5% (w/v) solution dissolved in 0.1M-potassium phosphate buffer, pH7.5.

15.1 Measurement of 'benzoate oxidate' in the oxygen electrode

Since there is no suitable method for measuring 'benzoate oxidase' (BO) in extracts, it was estimated by measuring oxygen uptake in intact bacteria. Bacteria were resuspended in 0.1M-potassium phosphate buffer, pH7.0 to A_{500} = approx. 30. The buffer (2.0ml), distilled water and bacterial suspension were pipetted into the incubation vessel to a total volume of 3ml. The perspex disc was placed in position and the endogenous rate of respiration measured. Benzoate was then added as 100µl of a 15mM solution adjusted to pH7.0. The rate of oxygen consumption measured after substrate addition was corrected for the endogenous rate of respiration.

16. Charcoal treatment of bovine serum albumin

The procedure of Chen (1967) was used for the removal of fatty acids from BSA. Albumin (5.0g) was dissolved in 50ml distilled water and 2.5g of washed, activated charcoal was added. The pH was lowered to 3.0 by addition of 5M-HCl. The solution was placed in an ice bath and mixed magnetically for 1h. The charcoal was removed by centrifugation at 20,000g for 20min at 2°C in the MSE Highspeed 18 centrifuge. The solution was then brought to pH7.5 by the addition of NaOH and made up to a final volume of 100ml with distilled water.

17. Melting point determination

A Gallenkamp melting point apparatus (Type MF-30; Gallenkamp, Portract Lane, Stockton-on-Tees, Teeside TS18 2PT) was used.

18. Optical rotation

Optical rotation was determined using a Perkin-Elmer Polarimeter (Boden Seeweric Perkin-Elmer & Co., GMBH/Überlingen) under the direction of Professor Capon (Department of Chemistry, Glasgow University). The light source was a sodium lamp (598nm) and the solution was placed in a 10cm glass tube. The specific rotation [α] was determined using the following equation:

$$[\alpha] = \frac{100 \times A}{c \times l}$$

where A is the observed rotation (+ or -) in degrees, c is the concentration in g/100ml and l is the length of the optical path through the solution, in decimeters.

19. pH measurement

The pH values of most solutions were determined using a direct reading pH meter (Model 7010; E.I.L. Ltd., Cumbernauld, Glasgow G67 1AG) connected to a combined glass electrode (224; Probion Ltd., Glenrothes, Fife KY6 3AE). The pH values of small volumes were measured with a pH meter (Model 2320; E.I.L.) fitted with an assembly containing microelectrodes.

20. Protein estimation

Protein contents of suspensions of intact bacteria were estimated on the assumption that a suspension of turbidity $A_{500} = 1$ contains 168μg protein ml^{-1} (Beggs, 1974).

Protein contents of extracts were measured by determining the A_{280} and then using the conversion factor determined by Fewson (unpublished results) when he compared the A_{280} values of a series of

extracts with the protein content based on the Lowry method (Lowry et al., 1951) using BSA as standard:

$$\text{protein content (mg ml}^{-1}) = \frac{A_{280}}{4.9}$$

The method of Lowry et al. (1951) was used to estimate the protein contents of membrane fractions using BSA as standard.

21. Statistical methods

Mean and standard deviation, the correlation coefficient (r) and the line of best fit were calculated by the method of least mean squares using either a Canon Canola 167P calculator (Block & Anderson, London) or a Texas TI-51-III calculator (Texas Instruments Ltd., Bedford MK41 7PU). All results are quoted as means $\pm$ the standard deviation, with the number of determinations in parentheses.

22. Glassware

22.1 General glassware

All growth flasks were cleaned before use by autoclaving in Hemosol solution ($10 g l^{-1}$; Meinecke & Co., Baltimore, U.S.A.). The glass was thoroughly rinsed with tap water and then with distilled water before drying in an oven. Other glassware was washed in Hemosol, rinsed and dried.

22.2 Pipettes

All pipettes were cleaned by soaking in 'Kirbychlor' disinfectant solution (H. & T. Kirby & Co. Ltd., Mildenhall, Suffolk) then in Hemosol solution, followed by thorough rinsing in tap and deionised water, and drying in an electrically heated pipette drier. All pipettes were plugged with cotton wool.

23. Safety

Bacterial cultures were killed by autoclaving before disposal and glassware washed as described in Methods 22.1. Any bacterial spillage was swabbed with propan 1-ol (10%).

Since NTG is a carcinogen appropriate precautions were observed. Another person stood by whilst all operations were carried out in a fume cupboard on a large sheet of aluminium foil, covered with paper towelling. Two pairs of Triflex gloves (Travenol Laboratories Ltd., Thetford, Norfolk) were worn, which were wrapped up in the foil when the treatment was finished. The 'foil parcel' was then autoclaved. A microspatula (kept only for this purpose) was used to transfer a few crystals of NTG to each plate. After use it was left overnight in a covered beaker containing dilute NaOH (about 2l). The spatula was then rinsed thoroughly and the beaker autoclaved.

All other precautions taken in the interest of safety are as described in the University of Glasgow Safety Handbook.

R E S U L T S

The results which follow are grouped into nine Sections. The order of presentation is not necessarily chronological, partly because the results were often obtained by refinements of methods suggested by other experiments described in different Sections.

Section 1 deals with the development of reliable methods for growing bacteria in good yield and with high specific activity of D-mandelate dehydrogenase. It includes experiments with mutants whose isolation is not described until Section 6, and the use of an enzyme assay whose development is presented in Section 3.

Section 2 identifies phenylglyoxylate as the sole product of D-mandelate oxidation. This confirmed other, less direct, evidence that the first step of D-mandelate metabolism involves a D-mandelate dehydrogenase.

The development of a reliable, sensitive and accurate spectrophotometric assay for D-mandelate dehydrogenase using cell-free extracts (Section 3) was one of the major problems of the developmental work. In the early stages, not only were the requirements for the assay system not known, but it was sometimes difficult to obtain sufficient bacteria for the measurement of activity (Section 1), and furthermore the bacteria had low specific activity of the enzyme because D-mandelate dehydrogenase turned out to be heat-labile.

An alternative, but indirect, method of estimating D-mandelate dehydrogenase activity was by measuring oxygen uptake with an oxygen electrode. Prior to the development of the spectrophotometric assay, the ability of various mutant strains (e.g. those described in Section 6) to utilize D-mandelate, and other substrates of the mandelate pathway, was determined using this technique with intact bacterial suspensions. The results presented in Section 4, however, include more recent work done with ultrasonically disrupted bacteria which bears on the mechanisms of electron transfer by D-mandelate dehydrogenase.

A preliminary comparison of evolved D-mandelate dehydrogenase and original L-mandelate dehydrogenase in mutants derived from A. calcoaceticus NCIB8250 is given in Section 5.

Probably the most significant set of results is in Section 6 which demonstrates that the regulation of the evolved D-mandelate dehydrogenase activity in mutants derived from NCIB8250 is co-ordinate with the other mandelate enzymes. Included in this Section is a description of the isolation of two classes of mutants which constitutively synthesize phenylglyoxylate carboxy-lyase. Since parts of the isolation technique had been developed previously (Fewson et al., 1978) these mutants were obtained early in the work, and mutants such as 219 and 123 (see Table 2 in Methods) proved useful in the experiments described in all the earlier Sections.

Section 7 describes the isolation of mutants from strain C48, an auxotroph of wild-type strain EBF65/65. This strain shows the opposite pattern to strain NCIB8250 since it metabolizes D-mandelate and not L-mandelate. The isolation of mutants able to utilize the L- isomer is described and the control of the evolved L-mandelate dehydrogenase and original D-mandelate dehydrogenase shown to be identical with each other.

A brief comparison of the original and the evolved D- and L-mandelate dehydrogenases in a mutant ultimately derived from strain NCIB8250 and one ultimately derived from strain EBF65/65 is given in Section 8.

Finally, a semi-quantitative study of the enrichment of mutants able to utilize D- or L-mandelate (Section 9) provides a basis for speculation concerning the evolution of the novel enzyme activities.

1. Development of methods for growth of bacteria

1.1 Problem of lysis

During the course of this work, especially during the early stages, persistent problems were encountered whilst growing strain 41 and mutants isolated from it. These bacteria were susceptible to lysis when grown in defined media e.g. under the conditions used for other strains by Beggs & Fewson (1977) where bacteria were first grown for 17h in 5mM-L-glutamate/salts medium and then inoculated (25%, v/v) into a further flask of the same medium. Lysis was recognised by a decrease in turbidity of the culture; in extreme cases the cultures cleared but usually growth recommenced after a while and was then normal (see e.g. Fig. 2). The fall in turbidity was accompanied by frothing, presumably due to denaturation of released bacterial protein as a result of stirring. Examination of the lyzed cultures by phase contrast microscopy revealed considerable bacterial clumping, as compared to the single or paired bacteria observed in the absence of lysis.

The occurrence of lysis was extremely variable and consequently difficult to study systematically. For instance in one experiment, nine flasks were set up under a variety of conditions but none of the cultures lyzed.

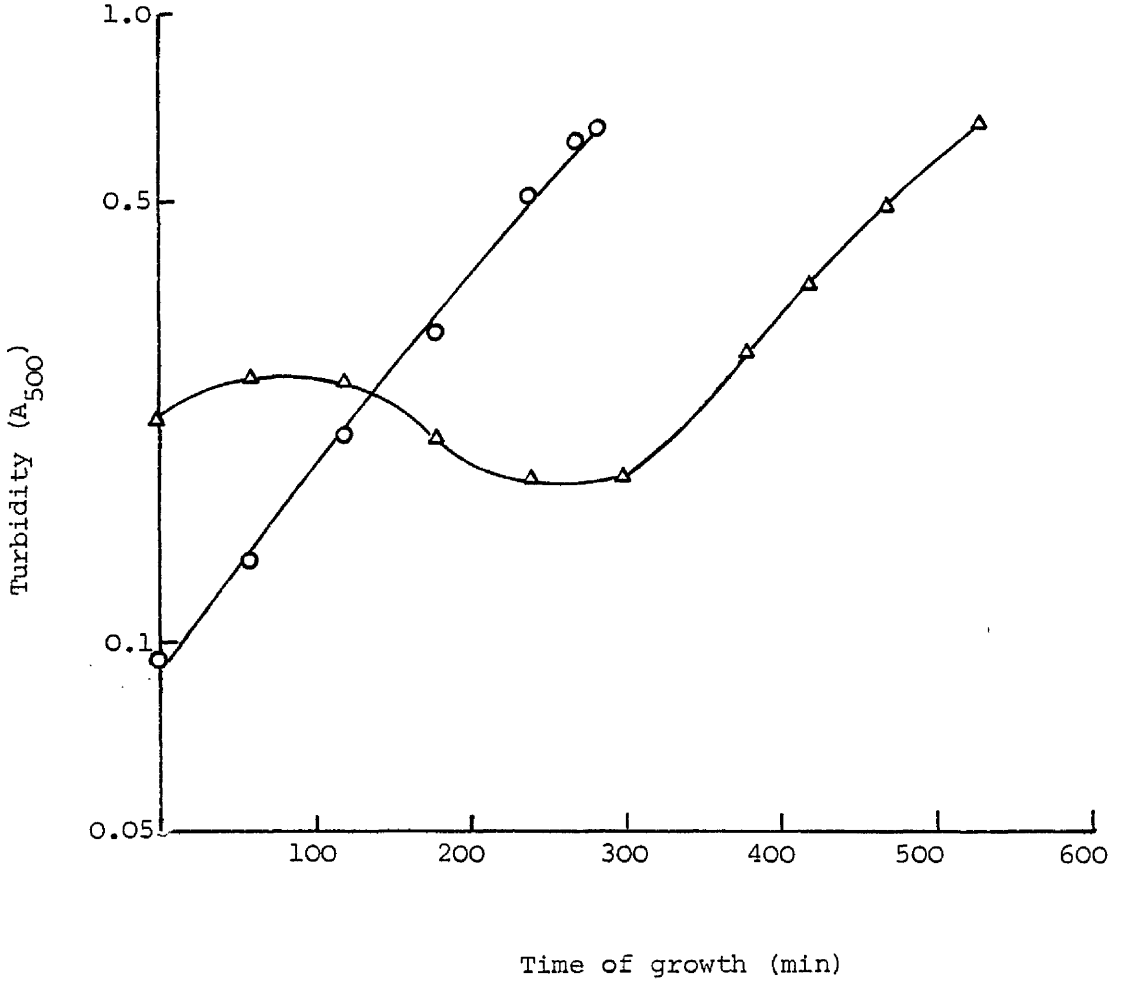
The possibility that a virus was the causative agent was investigated on one occasion. Samples of lyzed culture (and serial dilutions) were placed on nutrient agar and L-glutamate/salts agar plates which had been spread with strains 41 or NCIB8250 and grown for 24h. After a further 2d incubation at 30°C the plates were examined but no clear plaques were seen in the bacterial lawns.

Fig. 2 An example of lysis whilst growing mutant 219 in 5mM-L-glutamate/salts P2 medium

Nutrient broth cultures were grown at 23°C for 48 or 72h. These bacteria were inoculated (25%, v/v) into appropriate medium so that the final composition was 5mM-L-glutamate/salts P2 (in 400ml). The cultures were grown at 23°C (Methods 6 and 6.8) and at intervals samples were taken for turbidity measurements (Methods 5). The cultures were harvested when the turbidity reached A_{500} = approx. 0.66.

O : culture grown from a 48h nutrient broth inoculum

Δ : culture grown from a 72h nutrient broth inoculum


1.0
0.5
0.1
0.05
Turbidity (A_{500})
100
200
300
400
500
600
Time of growth (min)

The physical shearing of bacteria as a result of stirring was also studied because cultures grown on a rotary shaker never lyzed, even in defined media. The results of experiments in which cultures were stirred at different speeds suggested that fast stirring may cause or exacerbate lysis, and indeed it has been shown by Fewson (unpublished work) that even strains NCIB8250 and HMM5 lyze when grown at every high stirring speeds in a fermenter. Neither the rate at which air was passed over the culture, nor the pH of the culture seemed to affect lysis. Some results, however, suggested that lysis may be less likely to occur at 23°C than at 30°C. There is no evidence for lysis in normal static nutrient broth cultures.

In order to combat the problem of lysis, it was decided to grow bacteria for shorter times in defined media. To obtain reasonable yields, therefore, larger inocula were required and so nutrient broth cultures were grown to high turbidities (approximately $A_{500} = 1$) by incubating at 23°C for up to 72h or at 30°C for up to 48h; defined media were then inoculated using 25% (v/v) of these cultures. All the experiments on regulation of enzyme activity in A. calcoaceticus (Results 6) followed this procedure so that results with different strains could easily be compared. The later experiments on the properties of the mandelate dehydrogenases (Results 5) also involved this method of growth, even if strains NCIB8250, HMM5 and 123 were being used, since it was both reliable and convenient.

Despite all these precautions, lysis was still sometimes observed with mutant 219. No obvious pattern has yet been discerned;

for instance the fact that in the experiment illustrated in Fig. 2 the culture grown from a 72h nutrient broth inoculum lyzed whereas that from a 48h nutrient broth inoculum did not, is entirely fortuitous. In other experiments of this sort neither culture lyzed, or there was lysis of the culture grown from the 48h inoculum. It may be noted that the cultures illustrated in Fig. 2 were harvested when they reached a turbidity of A_{500} = approximately 0.66 and had specific activities of 103 and 160 $_{nmol}$ $min^{-1}(mg\ protein)^{-1}$ for D-mandelate dehydrogenase.

1.2 Temperature of growth

In all the early work bacteria were grown at 30°C, this temperature being optimal for yield and growth rate of A. calcoaceticus NCIB8250 (Fewson 1967a) and having been used for all previous work in the laboratory on the mandelate enzymes. However, it became evident that D-mandelate dehydrogenase activity following growth of mutants such as 148 and D40E was much lower than L-mandelate dehydrogenase activity in mutant HMM5 even when the optimal conditions for assay of D-mandelate dehydrogenase (see section 3) were used. When this observation was first made, the possibility of obtaining a higher specific activity by changing the growth rate was considered. In the course of a number of preliminary investigations, it was found that lowering the growth temperature to 23°C reduced growth rate and gave considerably greater activity of D-mandelate dehydrogenase for strains 148, 219 and D6. In addition some increase of L-mandelate dehydrogenase activity was noted for strain HMM5 grown at 23°C although this did not occur with strains D6 or 219. These results

are summarized in Table 6, and raised the possibility that D-mandelate dehydrogenase is relatively heat-labile compared to L-mandelate dehydrogenase, in other words that the effect on specific activity of D-mandelate dehydrogenase was caused by the effect of temperature on the enzyme and was not an indirect effect of temperature changing the growth rate and hence the enzyme specific activity. To test this possibility, the lability of D- and L-mandelate dehydrogenase activities was examined in both whole bacteria and extracts.

Mutant 219 was grown at 23°C to give high levels of D- and L-mandelate dehydrogenase activity. A washed bacterial suspension was then incubated at 23°C or 30°C, and the enzymes assayed at intervals (Fig. 3). The activity of L-mandelate dehydrogenase was relatively stable even at 30°C. On the other hand, the activity of D-mandelate dehydrogenase whilst declining slowly at 23°C, fell off rapidly at 30°C. The loss of activity displayed approximately first order kinetics and the time for reducing the original D-mandelate dehydrogenase activity to 50% ($t_{\frac{1}{2}}$) was 100min at 30°C.

Incubation of a mixture of extracts from mutants derived from strain 41 (see also Fig. 20 later) confirmed the relative heat-lability of the D-mandelate dehydrogenase. Again, approximately first order kinetics were observed for loss of activity with the following 'half-lives':

Temperature of incubation (°C)	$t_{\frac{1}{2}}$ (min)	
	DMDH	LMDH
23	600	>1440
30	140	>1440
45	10	40

Table 6 The effect of growth temperature on D- and L-mandelate dehydrogenase activity

Mutants HMM5, 148, 219 and D6 were grown in 5mM-L-glutamate/salts P2 medium at 23°C as described in Methods 6.5. The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted (Methods 9.1.1). Activities of D- and L-mandelate dehydrogenase were assayed in triplicate (Methods 11.1 and 11.2.1).

The specific growth rate (μ) was calculated by measuring the starting turbidity (Ao), the turbidity at harvest (At), and the time of growth (t), and using the following relationship:

$$\ln \frac{At}{Ao} = \mu t$$

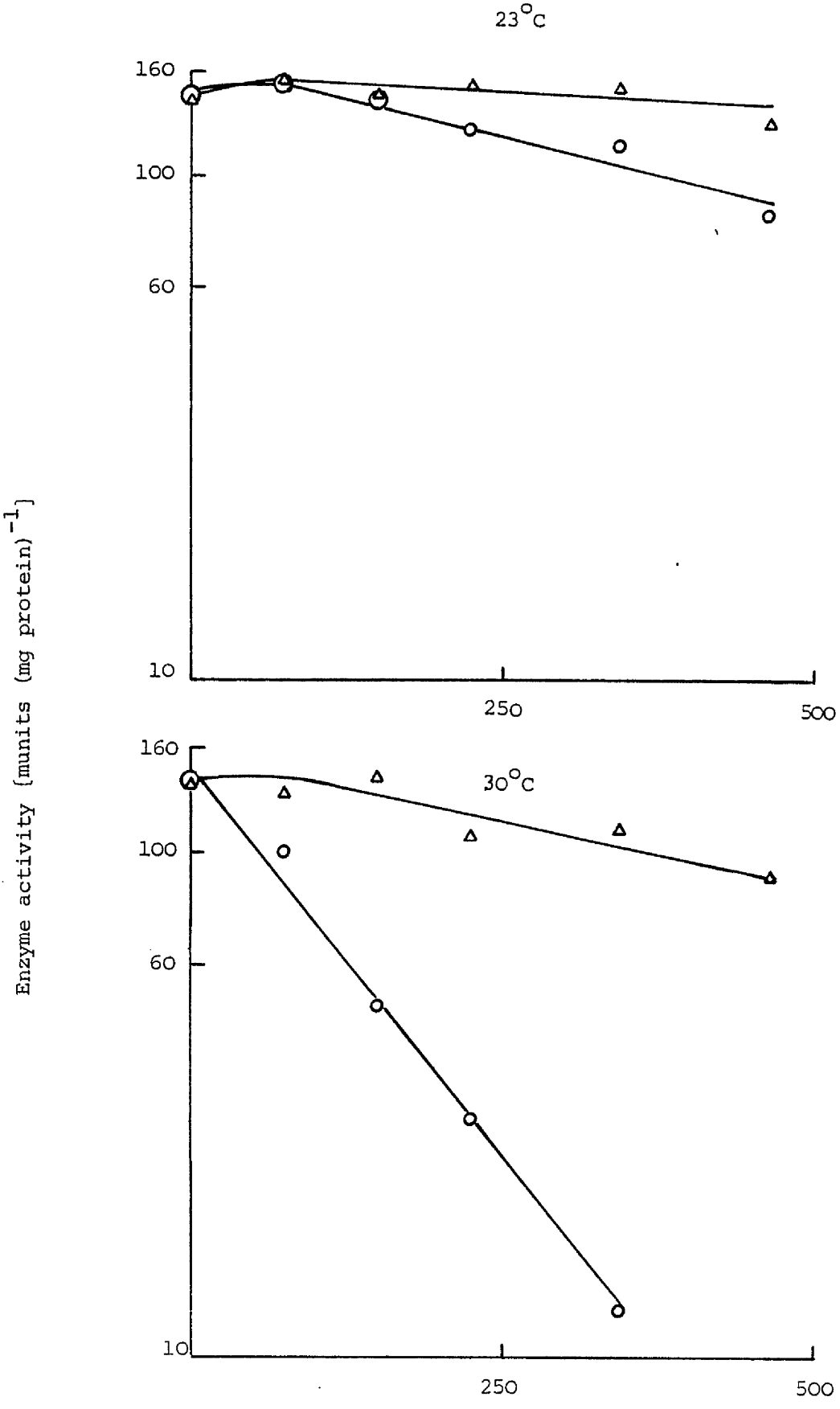
Mutant strain	Temp. of growth °C	μ (h^{-1})	Enzyme activities munits (mg protein)$^{-1}$	
			DMDH	LMDH
HMM5	23	0.47	0.5	147
	30	0.74	0.2	89
148	23	0.43	52	1
	30	0.68	15	2
219	23	0.34	130	113
	30	0.59	36	144
D6	23	0.45	131	133
	30	0.71	35	135

Fig. 3 Heat inactivation of D- and L-mandelate dehydrogenase in suspensions of intact bacteria

Mutant 219 was grown at 23°C in 5mM-L-glutamate/salts P2 medium (Methods 6.8). The bacteria were harvested, washed and stored as described in Methods 7.1. The bacteria were resuspended to A_{500} = 4.5 in 0.1M-potassium phosphate buffer, pH7.5 and incubated, with stirring at 23°C or 30°C (54ml in 100ml conical flasks). Samples (5ml) were taken at invervals and the bacteria were ultrasonically disrupted and centrifuged as described in Methods 9.1.1. The activities of D- and L-mandelate dehydrogenase were determined in triplicate in each extract (Methods 11.1 and 11.2.1). Values below 10munits $(mg\ protein)^{-1}$ have not been included.

O : D-mandelate dehydrogenase

Δ : L-mandelate dehydrogenase


23°C
160
100
60
10
250
500
30°C
160
100
60
10
250
500
Enzyme activity (munits (mg protein)$^{-1}$)
Time of incubation (min)

These results show that D-mandelate dehydrogenase is indeed heat-labile and this probably explains the low activities recovered following growth at 30°C. Strains possessing D-mandelate dehydrogenase activity were subsequently grown at 23°C.

1.3 Constituents of growth medium

When bacteria were grown in salts medium containing L-glutamate, an increase in pH from 7.0 to 7.7 was observed; this is probably due to the formation of ammonia during glutamate metabolism. In order to reduce the extent of this pH change the buffering capacity of the medium was increased by doubling the phosphate concentration to 4gKH_2PO_4 per litre (see 'salts P2 medium' Methods 4.1). Furthermore when growing on L-glutamate no additional source of nitrogen was deemed necessary and so the $(NH_4)_2SO_4$ was omitted, thus giving 'basal P2 medium' which contains only 4gKH_2PO_4 per litre.

2. The product of D-mandelate oxidation

When the D-mandelate utilizing strains D40E ($L^{O}D^{i}P^{i}$) and 148 ($L^{O}D^{C}P^{C}$) were grown on medium containing D-mandelate, the medium acquired the distinctive odour of benzaldehyde. A hexane extract of the culture gave the same absorption spectrum (λmax. = 240nm) as authentic benzaldehyde. This suggested that D-mandelate was being metabolized via the same pathway as L-mandelate (Scheme 2). If this was so, a possible first step in the metabolism of D-mandelate would be D-mandelate $\rightarrow$ phenylglyoxylate, and it should be possible to observe accumulation of phenylglyoxylate under conditions where decarboxylation to benzaldehyde did not occur. Preliminary experiments showed that D-mandelate oxidising ability could be separated from phenylglyoxylate carboxy-lyase activity by high speed centrifugation (100,000g for 2.5h; Methods 9.2). The membrane fraction contained about 70% of the D-mandelate oxidising ability, whereas the soluble fraction contained about 70% of the phenylglyoxylate carboxy-lyase activity. Phenylglyoxylate carboxy-lyase activity is dependent upon the inclusion of TPP in the assay mixture (Methods 11.3). When phenylglyoxylate carboxy-lyase was measured without exogenous TPP no activity was detected in the membrane fraction and there was a 14-fold reduction of activity in the soluble fraction. Thus little or no decarboxylation of phenylglyoxylate would be expected in a membrane preparation in the absence of added TPP.

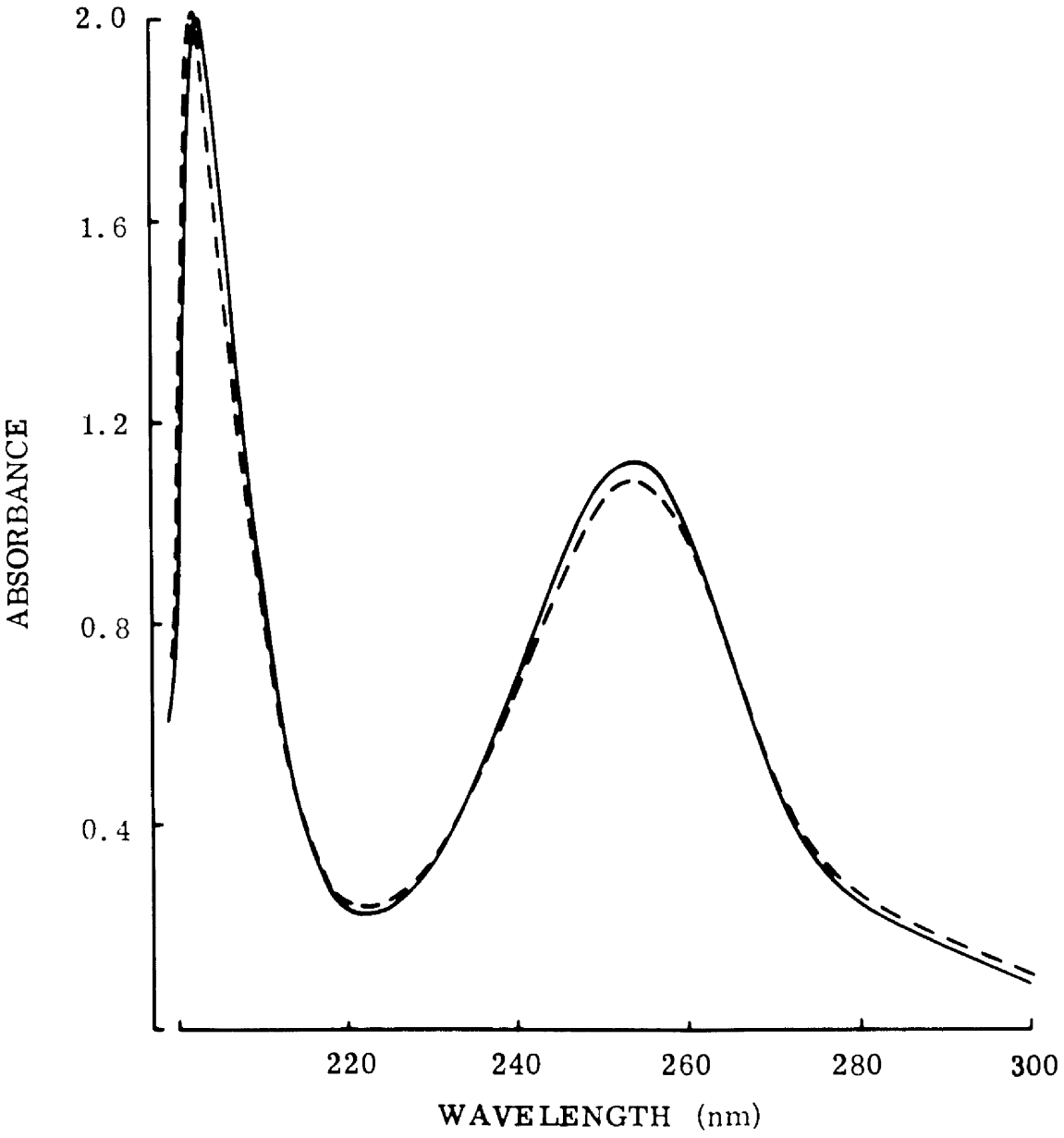
A membrane fraction was therefore shaken with D-mandelate and PMS at 23^{O}C as described in the legend to Fig. 4. Samples were

Fig. 4 Absorption spectra of phenylglyoxylate and the reaction product of the oxidation of D-mandelate

Mutant 219 was grown in 5mM-L-glutamate/salts P2 medium at 23°C as described in Methods 6.8. The bacteria were harvested, washed, stored (Methods 7.1), and then ultrasonically disrupted and a membrane fraction prepared (Methods 9.2). The membrane fraction (approx. 13.7mg protein) was incubated with 0.33mM PMS and 2mM-D-mandelate (final concn.; total volume 10ml) at 23°C with shaking (73 oscillations min^{-1}). Samples (1ml) were taken at intervals and treated with perchloric acid, extracted with chloroform, then with NaOH, and finally acidified with HCl (Methods 12.1). An authentic solution of phenylglyoxylate was subjected to the same extraction procedure. The absorption spectra of these extracts were then measured.

———— sample

– – – – – – authentic phenyglyoxylate


2.0
1.6
1.2
0.8
0.4
ABSORBANCE
220
240
260
280
300
WAVELENGTH (nm)

taken at intervals for both qualitative and quantitative estimation of phenylglyoxylate. PMS was included in the reaction mixture in order to enhance the rate of D-mandelate oxidation (see results for oxygen uptake, Table 12).

2.1 Identification of phenylglyoxylate as the reaction product

The absorption spectrum of a chloroform extract of a reaction mixture in which D-mandelate has been oxidized was identical with that of a similar extract of a solution of authentic phenylglyoxylate ($\lambda max.$ = 253nm, $\lambda min.$ = 222nm; Fig. 4).

Phenylglyoxylate standards and a sample prepared by chloroform extraction of a reaction mixture (legend to Fig. 4) were applied as solutions in diethyl ether to Silufol sheets for thin layer chromatography and the chromatograms were developed (Methods 13). The R_F value for the phenylglyoxylate standard was 0.346 $\pm$ 0.168 (8). The sample from the reaction mixture gave only one spot with an R_F value of 0.337 $\pm$ 0.012 (5), and only one spot was obtained when sample and authentic phenylglyoxylate were co-chromatographed. In a separate experiment benzoate was shown to have an R_F value of 0.113.

2,4-Dinitrophenylhydrazones were prepared from phenylglyoxylate, phenylpyruvate, pyruvate and the chloroform extract of the reaction mixture (Methods 14). These yellow compounds were separated by paper chromatography (Methods 14) and the dinitrophenylhydrazone of phenylglyoxylate ran in the same position as the dinitrophenylhydrazone of the product of D-mandelate oxidation:

R_F for phenylglyoxylate 0.802 for two determinations

R_F for phenylpyruvate 0.894

R_F for pyruvate (four spots) 0.231, 0.435, 0.696, 0.966

R_F for sample 0.795

R_F for sample + phenylglyoxylate (one spot) 0.788

Fig. 5 shows the absorption spectra of these dinitrophenylhydrazones. The spectra of the sample (λmax. = 388nm, λmin. = 310nm) or a mixture of the dinitrophenylhydrazones of sample and of authentic phenylglyoxylate (λmax. = 388nm, λmin. = 307nm) are very similar to that of phenylglyoxylate (λmax. = 385nm, λmin. = 307nm). The slight shift in absorbance at higher wavelengths for the sample may be due to some component(s) of the reaction mixture not precipitated by perchloric acid.

2.2 Quantitative estimation of the phenylglyoxylate produced by D-mandelate oxidation

The accumulation of phenylglyoxylate during the oxidation of 20µmol of D-mandelate was monitored over 4h (Fig. 6). Accumulation was approximately linear for the first 20 min, by which time 17µmol phenylglyoxylate had been formed. The amount of phenylglyoxylate continued to increase until, after a total time of about 2h, 20µmol phenylglyoxylate had been produced. This amount was not significantly altered during the subsequent 2h period.

The stoicheiometric equivalence of D-mandelate and phenylglyoxylate indicates that this is the only pathway involved in D-mandelate

Fig. 5 Absorption spectra of 2,4-dinitrophenylhydrazones

2,4-Dinitrophenylhydrazones were prepared as described in Methods 14. The final solutions were in 10% (w/v) Na_2CO_3. Absorption spectra were measured against a reference solution of 10% (w/v) Na_2CO_3.

———————— sample

—— —— —— pyruvate

—·—·— phenylpyruvate

- - - - - - phenylglyoxylate

..... phenylglyoxylate + sample

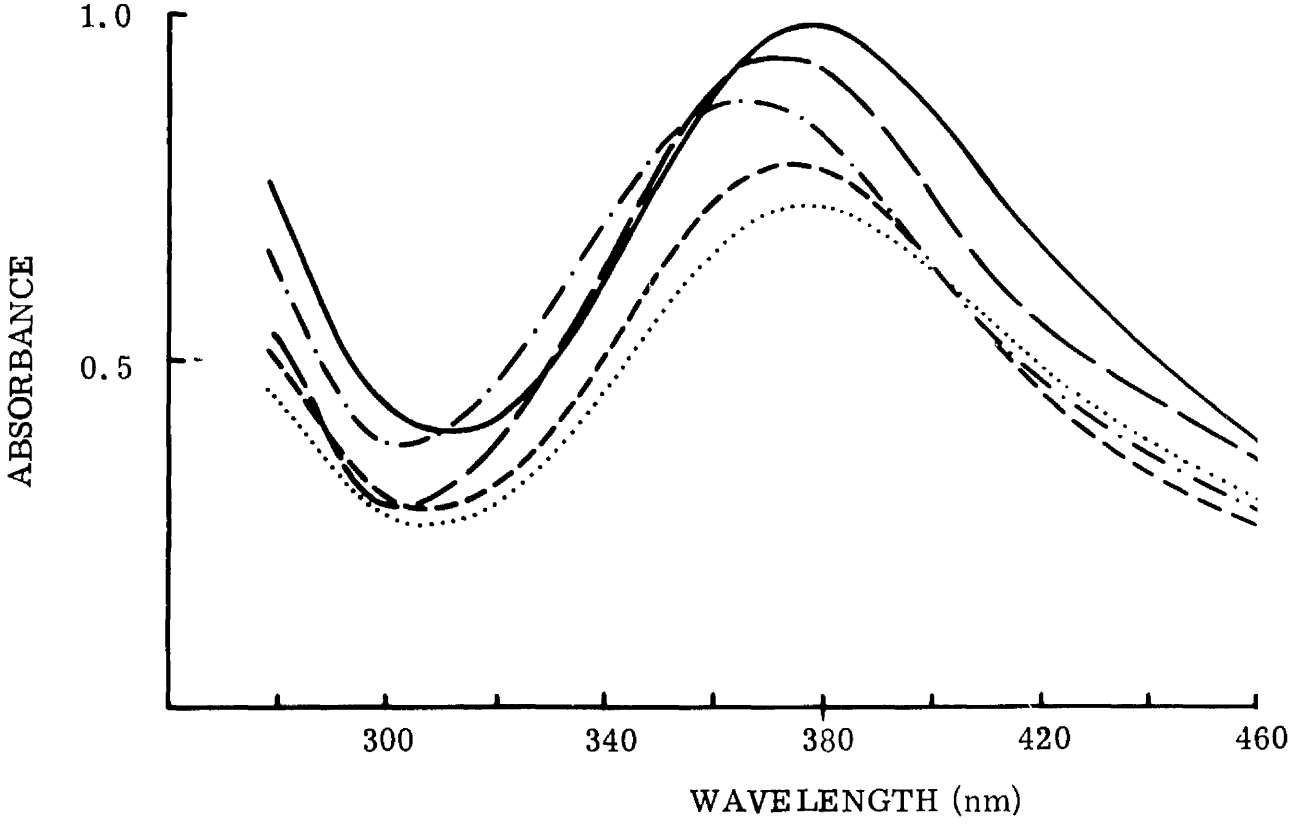
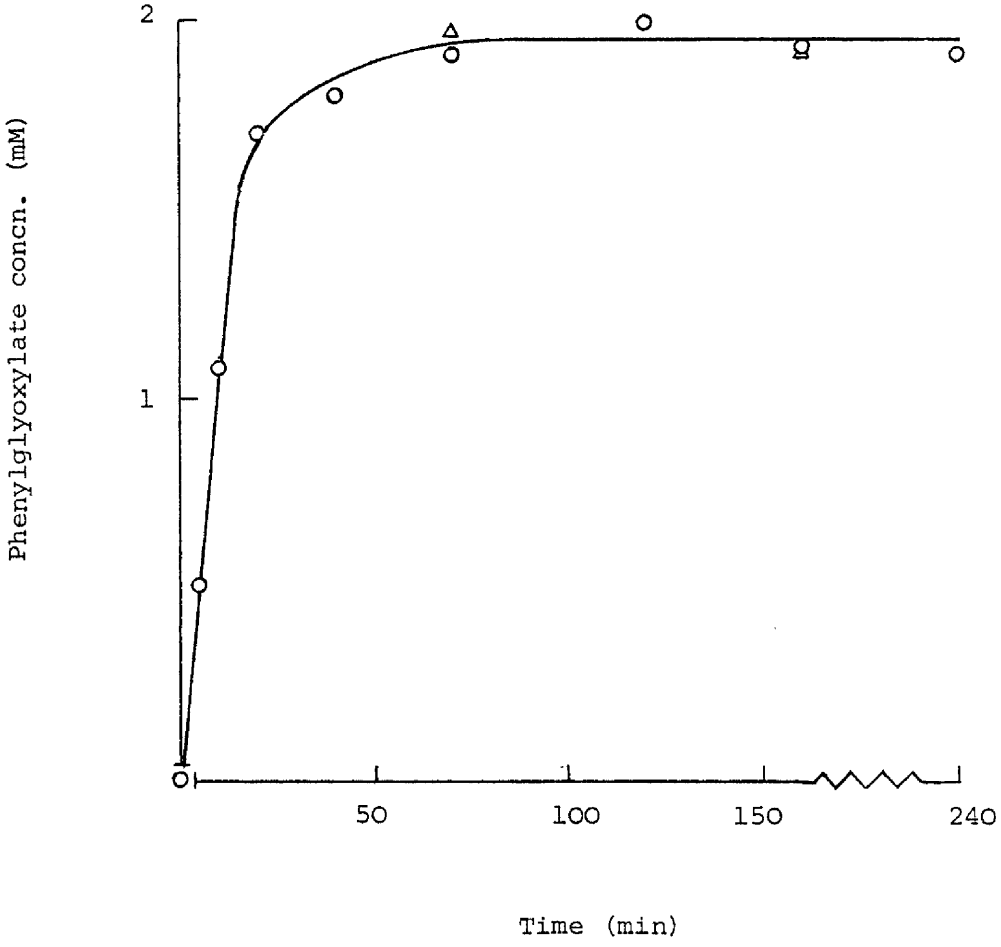

1.0
0.5
ABSORBANCE
300
340
380
420
460
WAVELENGTH (nm)

Fig. 6 Formation of phenylglyoxylate from D-mandelate by membrane preparations of mutant 219

See Legend to Fig. 4. The absorbance of each sample was measured at 253nm and the concentration of phenylglyoxylate calculated by reference to a standard curve (Methods 12.1, Fig. 1). Duplicate 10ml reaction mixtures were set up (O, Δ).


Phenylglyoxylate concn. (mM)
2
1
50
100
150
240
Time (min)

metabolism. It would seem reasonable to propose that the conversion of D-mandelate to phenylglyoxylate is mediated by a D-mandelate dehydrogenase analogous to the dehydrogenation of L-mandelate to phenylglyoxylate.

3. Development and assessment of a method for assaying D-mandelate dehydrogenase in extracts

In order to study the regulation of D-mandelate metabolism, and in order to characterize the enzyme involved, a method of quantifying D-mandelate dehydrogenase activity was needed. Initially, oxygen electrodes were used to monitor uptake by intact bacteria utilizing D-mandelate. This gave some useful preliminary results but the method is time consuming and unsatisfactory for quantitative measurements; furthermore an assay system using extracts was needed for many of the experiments we had in mind.

3.1 Ultrasonic disruption of bacteria for the measurement of enzyme activity

The first requirement was for a reproducible method of extracting the enzyme from whole cells. Ultrasonic disruption was chosen, largely because this method has been used successfully for several enzymes of the mandelate pathway in A. calcoaceticus NCIB8250 (Livingstone & Fewson, 1972). An advantage of preparing an extract by ultrasonic disruption would therefore be that several other enzyme activities could be measured with one extract. This would be especially important when comparing the properties of D- and L-mandelate dehydrogenase (see Results 5).

The procedure developed by Livingstone (Livingstone, 1970; Livingstone & Fewson, 1972) had the disadvantage that large amounts of bacteria were required; they were resuspended to A_{500} = 30-50 and then 5ml amounts ultrasonically disrupted. Preliminary experiments, however, showed that the same D-mandelate dehydrogenase specific activity could be obtained by resuspending the bacteria to A_{500} = 5 and so less bacteria were required.

From a consideration of the results presented in Table 7 . in which the current and time of ultrasonic disruption were varied, the optimum conditions for extraction of D-mandelate dehydrogenase appeared to be 3.5min disruption at a current of 3A and these conditions were used in future work (Methods 9.1). Under these conditions specific and total activities of D- and L-mandelate dehydrogenase were maximal, lower activities being obtained with gentler disruption procedures, whilst more vigourous disruption led to loss of activity. The results for the mandelate dehydrogenases contrast with those for phenylglyoxylate carboxy-lyase, a soluble enzyme, whose activity was little affected over the range of conditions studied.

These results were obtained using soniprobe no. 3 which was generally used throughout this work. A similar pattern of results was obtained with proble no. 1, although the activity of D- and L-mandelate dehydrogenase was lower in every case, and much less protein was released.

Potassium phosphate buffers (pH7.0-8.0) and Tris-HCl buffers (pH8.0-9.0) were tested for both extraction and assay of D-mandelate dehydrogenase. Maximum enzyme activities were obtained using phosphate buffer, pH7.4 to 7.8. Subsequently, potassium phosphate buffer pH7.5 was routinely used for extraction and assay.

3.2 Development of an assay procedure for D-mandelate dehydrogenase

Initial attempts to measure D-mandelate dehydrogenase activity used the assay system developed for L-mandelate dehydrogenase (Livingstone & Fewson, 1972). This involves the use of the artificial electron acceptor DCIP, the reduction of which is followed spectrophotometrically. However only very low activities (less than 10munits (mg protein)$^{-1}$)

Table 7 Effect of current and time of ultrasonic disruption on release of enzyme activity

Mutant 219 was grown in 5mM-L-glutamate/salts P2 medium at 23°C as described in Methods 6.8. The bacteria were harvested, washed, stored (Methods 7.1) and then resuspended to A_{500} = 5 in 0.1M-potassium phosphate buffer, pH7.5. Samples (5ml) were ultrasonically disrupted using probe number 3 for a total time of either 2, 3.5 or 5min at the appropriate current. The current was switched off during alternate 30s intervals to allow cooling. After centrifugation the extracts obtained were assayed for D- and L-mandelate dehydrogenase (Methods 11.1 and 11.2.1) in triplicate and for phenylglyoxylate carboxy-lyase (Methods 11.3) in duplicate, as well as for protein content (Methods 20).

Setting	Current (A)	Time of disruption (min)	Protein content of extract (mg ml^{-1})	Enzyme activity: munits (mg protein)$^{-1}$ DMDH	LMDH	PC	Enzyme activity: Total units per ml DMDH	LMDH	PC
5	2.2	2	0.663	77	89	466	5.1	5.9	30.9
	2.2	3.5	0.714	91	120	444	6.5	8.6	31.7
	2.2	5	0.786	117	144	401	9.2	11.3	31.5
6	2.7	2	0.714	98	132	433	7.0	9.4	30.9
	3.0	3.5	0.775	133	182	403	10.3	14.1	31.2
	3.0	5	0.826	106	134	410	8.7	11.1	33.9
7	4.5	2	0.790	15	9	469	1.2	0.7	37.1
	4.9	3.5	0.796	0	2	431	0	0.2	34.3

could be measured by this procedure, although the growth rate implied appreciable activity *in vivo* [of the order of 100munits (mg protein)$^{-1}$]. There were also reasonable rates of oxygen uptake [500nmol O_2 min^{-1} (mg protein)$^{-1}$] in the oxygen electrode using intact bacteria with D-mandelate as substrate. This suggested that DCIP was a poor electron acceptor for the D-mandelate dehydrogenase. A search for alternative electron acceptors for the L-mandelate dehydrogenase was undertaken in the hope that these would prove effective with the D-mandelate dehydrogenase. Neither cytochrome c (0.1-0.4%, w/v) nor potassium ferricyanide (0.1-0.5mM) could substitute for DCIP as an acceptor. In the presence of PMS, the tetrazolium salt MTT [3-(4',5'-dimethylthiazoyl-2-yl)-2, 4-diphenyltetrazolium bromide; 0.23mM] gave about 80% of the activity measurable with DCIP alone, but none of the other tetrazolium salts (0.1 or 1mM) tested gave any activity [i.e. INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride; TTC, 2,3,5-triphenyltetrazolium chloride; NBT, 2,2'-di(*p*-nitrophenyl)-5, 5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride]. Addition of NAD^+ (0.1-4mM), $NADP^+$ (0.03-0.2mM), FAD (0.02-0.2mM) or FMN (0.02-2mM) did not increase enzyme activity in the presence of DCIP, and there was no activity with NAD^+ or $NADP^+$ in the absence of DCIP. Only PMS in the presence of DCIP gave an enhanced enzyme activity (see Fig. 12 later in Results). When PMS and DCIP were used to measure D-mandelate dehydrogenase, however, there was still no enhancement of activity.

Another possible reason for obtaining low activity was that the D-mandelate dehydrogenase might be inactivated during ultrasonic treatment. With this in mind, the effect of adding protein to the bacterial suspension before disruption was examined. When BSA was

included in the disruption buffer some increase in D-mandelate dehydrogenase activity was found using the original conditions for measuring L-mandelate dehydrogenase, i.e. with DCIP but no PMS. A striking increase in D-mandelate dehydrogenase activity in the presence of BSA was subsequently observed when PMS was also included in the reaction mixture. Further work demonstrated that BSA was equally effective when it was included solely in the reaction mixture, and that it was not needed during the disruption of the bacteria. Charcoal-treated BSA (Chen, 1967), which is relatively free from fatty acids, gave similar results to untreated BSA. The activities obtained when the concentration of PMS was varied in the presence of 10mg BSA per assay (3.33mg ml^{-1}), and the concentration of BSA varied in the presence of 1μmol PMS per assay (0.33mM) are shown in Fig. 7.

Subsequently 1μmol PMS and 10mg BSA were included in the 3ml reaction mixture and this was the standard assay system (Methods 11.1) used in the rest of the thesis.

Fig. 8 confirms the very large increase in enzyme activity obtained by including BSA and PMS in the reaction mixture. The activity measured in the presence of BSA was linear with respect to the amount of extract protein, but in the absence of BSA the apparent specific activity increased with increasing amounts of protein and D-mandelate dehydrogenase was barely detectable with small amounts of extract.

An additional advantage of adding BSA to the reaction mixture is that the reaction proceeds linearly with respect to time for at least 5min. In the absence of BSA the rate of reaction slows down after approximately 1min as shown below. The fall in rate is the

Fig. 7 The effect of PMS and BSA on the activity of D-mandelate dehydrogenase

Mutant 148 was grown in 5mM-D-mandelate + 5mM-L-glutamate/salts medium at 30°C (Methods 6.7). The bacteria were harvested, washed, stored and ultrasonically disrupted as described in Methods 7.1 and 9.1.1. D-Mandelate dehydrogenase activity was measured in the extract. In addition to enzyme, the assay mixtures contained (total volume 3.0ml):

(a) 200µmol potassium phosphate buffer, pH7.5

200nmol DCIP

appropriate concentrations of PMS

10mg BSA (dissolved in 0.2ml 0.1M-potassium phosphate buffer, pH7.5)

1.5µmol D-mandelate (adjusted to pH7.5) to initiate the reaction

(b) 200µmol potassium phosphate buffer, pH7.5

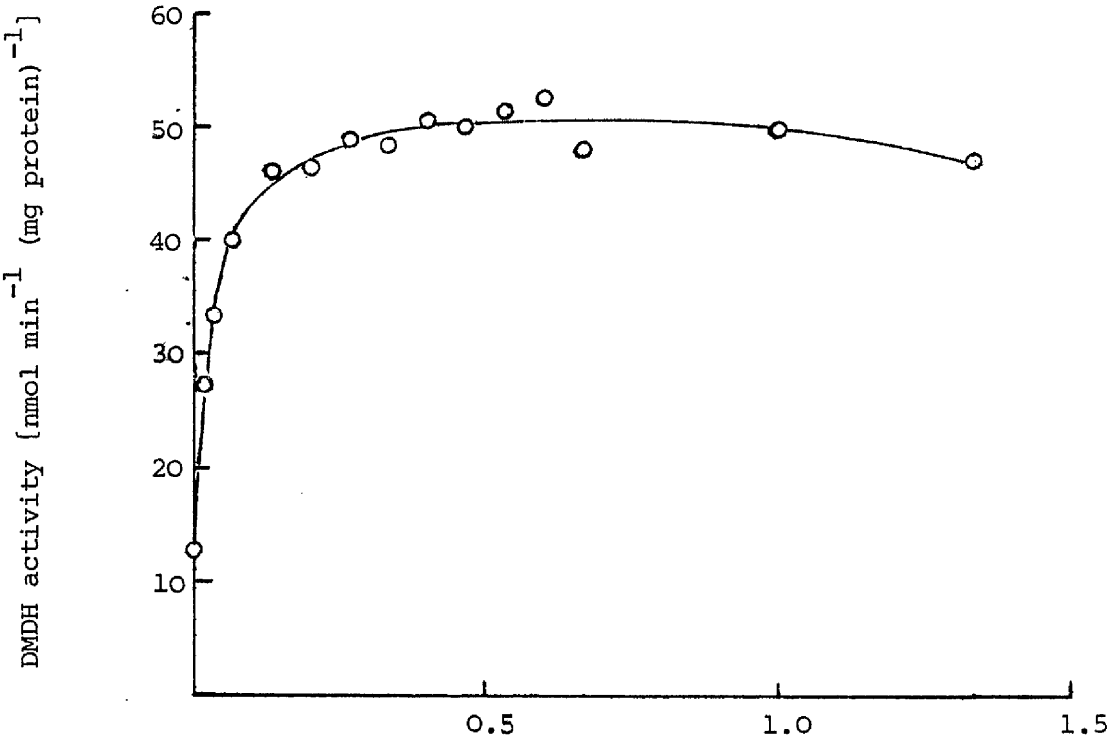
200nmol DCIP

1µmol PMS

appropriate concentrations of BSA (dissolved in 0.1M-potassium phosphate buffer)

1.5µmol D-mandelate (adjusted to pH7.5) to initiate the reaction

(a)


DMDH activity (nmol min^{-1} (mg protein)$^{-1}$)
60
50
40
30
20
10
0.5
1.0
1.5
PMS concn. (mM)

(b)

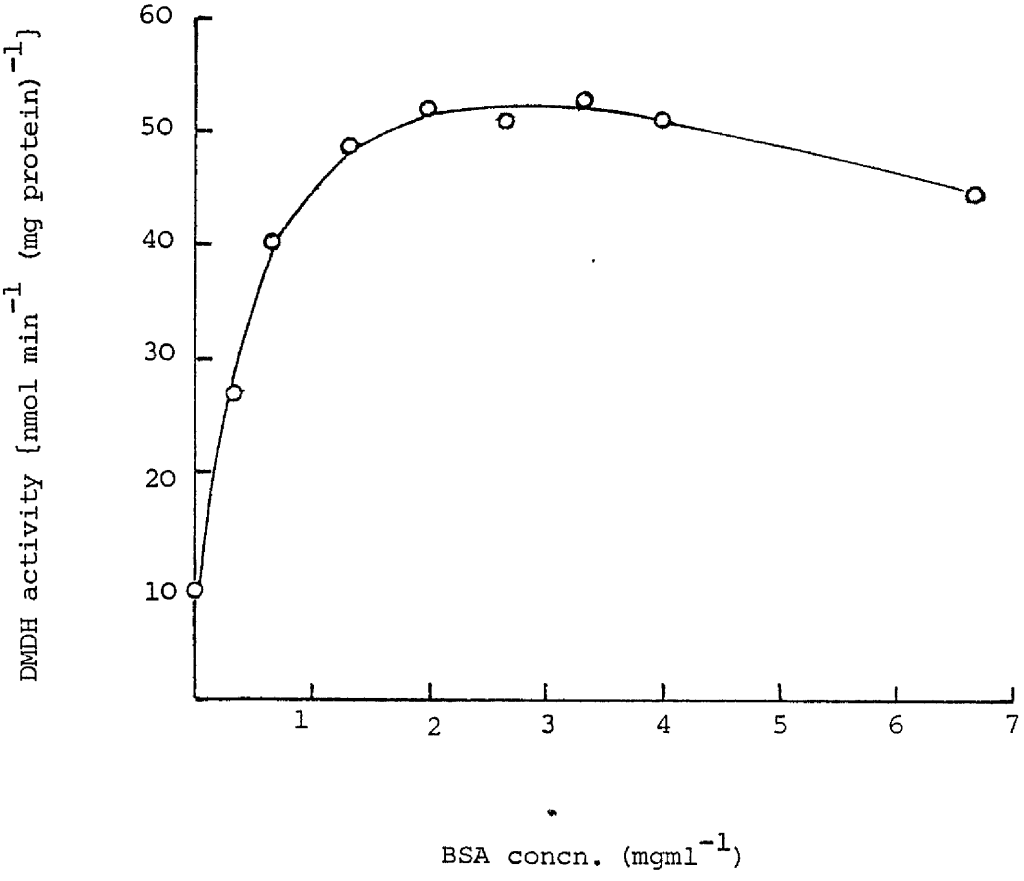
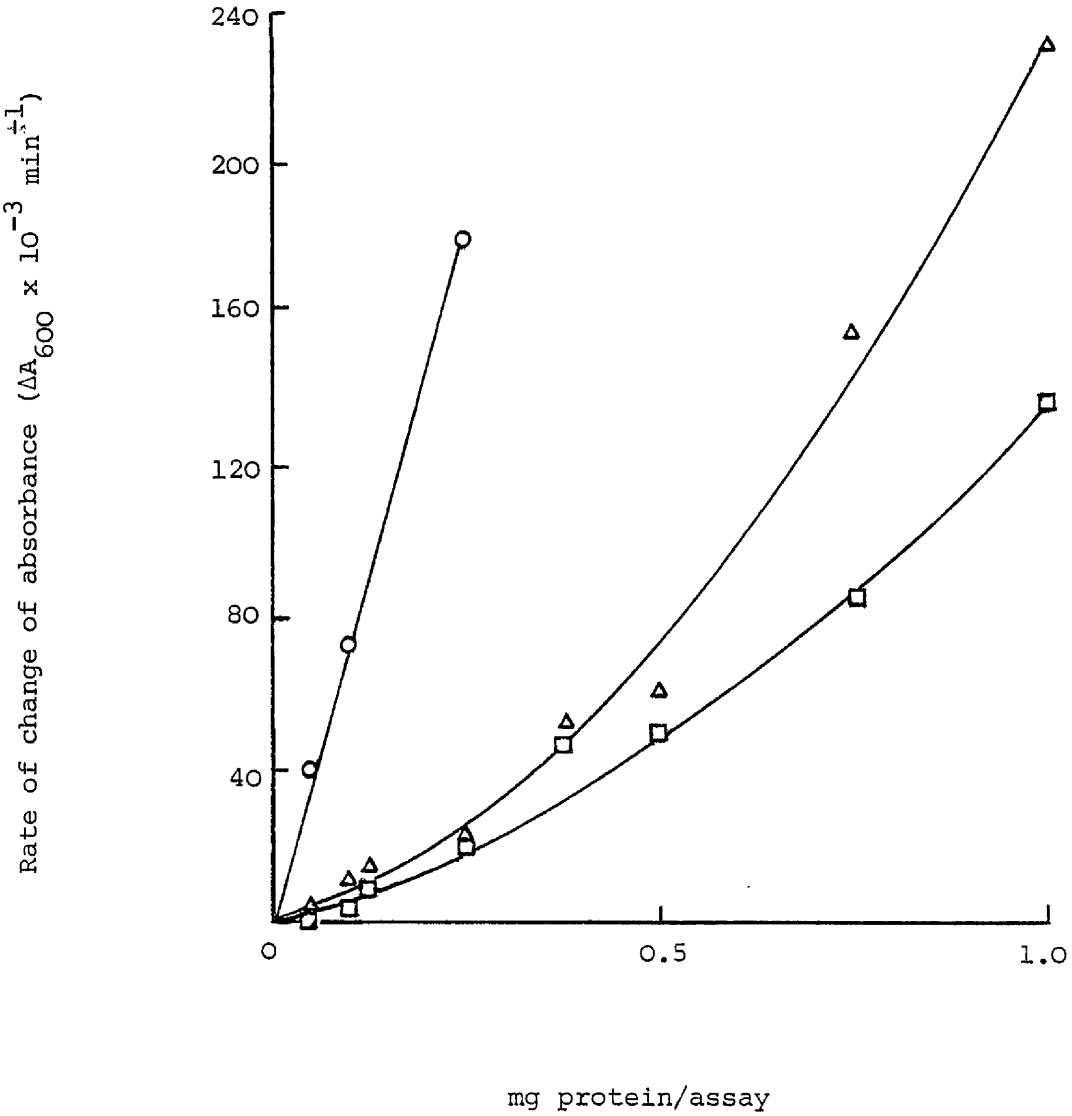

DMDH activity (nmol min^{-1} (mg protein)$^{-1}$)
60
50
40
30
20
10
1
2
3
4
5
6
7
BSA concn. (mgml^{-1})

Fig. 8 The effects of increasing protein concentration and of the omission of BSA and PMS from the reaction mixture used to measure D-mandelate dehydrogenase activity

Mutant 219 was grown in 5mM-L-glutamate/salts P2 medium at 23°C as described in Methods 6.8. The bacteria were harvested, washed, stored (Methods 7.1) and then a bacterial suspension was ultrasonically disrupted in a Rosett cell (Methods 9.1.2). D-Mandelate dehydrogenase activity was measured using the complete assay system (O; Methods 11.1) or omitting BSA (Δ) or BSA and PMS (□) from the reaction mixture.


240
200
160
120
80
40
0
0.5
1.0
Rate of change of absorbance (ΔA_{600} x 10^{-3} min^{-1})
mg protein/assay

same in the presence or absence of PMS. This decrease in rate was measured for D-mandelate dehydrogenase using a high concentration of protein to obtain measurable rates.

Time after initiation of assay (min)	Rate of reaction as % of initial rate			
	DMDH + PMS + BSA	DMDH + PMS no BSA	DMDH no PMS no BSA	LMDH no PMS no BSA
0 - 1	100	100	100	100
1 - 2	100	83	85	100
2 - 3	100	66	65	88
3 - 4	100	50	40	70

Non-linearity is also observed when measuring L-mandelate dehydrogenase activity using the original assay procedure of Livingstone (1971), see above. The decrease in rate is less pronounced than for the D-mandelate dehydrogenase and linearity is observed for approximately 2min. For this reason the initial rate of reaction (i.e. the first 2min) is always measured when calculating L-mandelate dehydrogenase activity.

The requirements for the various components of the D-mandelate dehydrogenase assay system are summarized in Table 8. An important feature is the endogenous activity measured without substrate. In all experiments, a control assay without substrate was included and this endogenous rate subtracted when calculating enzyme activities. In contrast, the original L-mandelate dehydrogenase assay gives a negligible endogenous rate in the absence of substrate; this is confirmed by the low activity measured in the absence of PMS and BSA, although the pH value of the reaction mixture shown in Table 8

Table 8 The effect of omitting reagents from the reaction mixture used to measure D-mandelate dehydrogenase

Mutants 123 and HMM5 were grown in 5mM-L-glutamate/salts P2 medium at 23°C and 30°C respectively (Methods 6.8 and 6.9). The bacteria were harvested, washed, stored, and ultrasonically disrupted as described in Methods 7.1 and 9.1.1. D-Mandelate dehydrogenase activity was then assayed in various reaction mixtures using the extract of mutant 123. In one case (bottom line) extract prepared from HMM5 was used to replace extract of 123. Each value is the mean of duplicate determinations.

Reaction component(s) omitted	D-mandelate dehydrogenase activity munits (mg protein)$^{-1}$
none	77
D-mandelate	14
BSA	8
PMS	13
PMS, BSA	3
PMS, BSA, D-mandelate	0.6
Extract	10
Extract of HMM5 replacing extract of 148	13

was 7.5 rather than 7.0 as used in the assay, but this is not significant. No D-mandelate dehydrogenase activity was measured if an extract of mutant HMM5 was used, although an L-mandelate dehydrogenase activity of 105munits (mg protein)$^{-1}$ was measurable with this extract. This confirmed that the activity measured was in fact due to a specific D-mandelate dehydrogenase since the only way in which mutant HMM5 differs from mutant 123 is in being able to grow on L- rather than D-mandelate.

D-Mandelate dehydrogenase was also assayed in Thunberg cuvettes which had been successively evacuated and filled with oxygen-free nitrogen at least six times. Oxygen-free nitrogen was also bubbled through the buffer and water, which make up the bulk of the reaction mixture, prior to their addition to the cuvettes. There was no difference in the rate of reaction under these anaerobic conditions as compared to aerobic assays done at the same time. Furthermore, only very low activity of D-mandelate dehydrogenase was obtained under both anaerobic and aerobic conditions when either BSA alone, or BSA + PMS was omitted from the reaction mixtures. These results suggest that oxygen did not interfere with the assay and so all subsequent assays were done under aerobic conditions.

3.3 Effect of the activity of the extract on the measurement of D-mandelate dehydrogenase

Since mutant strains might show a considerable variation in D-mandelate dehydrogenase activity, it was necessary to demonstrate that a range of enzyme specific activities could be measured accurately. To obtain samples having different specific activities of D-mandelate dehydrogenase, extract of strain 123, containing D-mandelate dehydrogenase activity, was diluted to different extents with extract of

strain HMM5, which has no D-mandelate dehydrogenase activity but has high L-mandelate dehydrogenase activity. When these samples were assayed (Fig. 9) the measured activities of D-mandelate dehydrogenase were linear over the range from 0 to 80munits (mg protein)$^{-1}$. This confirmed the reliability of the assay system both with respect to its ability to measure specific activities accurately and to the lack of interference by L-mandelate dehydrogenase.

3.4 The effect of extract volume on the measured D-mandelate dehydrogenase activity

Enzyme activities for different volumes of extract prepared from mutant 219 were measured in the presence and absence of substrate. The results (Fig. 10) show a good linear dependence of activity on extract volume when substrate was present. Measured activity in the absence of substrate was virtually constant confirming that this endogenous rate noted previously (Table 8) is independent of the presence of extract.

The relatively large standard deviation for volumes of 75µl probably reflects the fact that three Eppendorf pipettes (50µl, 20µl and 5µl volumes) were required to dispense this volume.

Subsequent to this work, a volume of 100µl of extract was used in all D-mandelate dehydrogenase assays. This volume, which was dispensed in a single pipetting operation, gave easily measurable rates, with low standard deviations, over a wide range of extract activities. Usually the assay was done in triplicate, with a fourth reaction mixture lacking substrate to give the control endogenous rate.

Fig. 9 Effect of specific activity of extract on the measurement of D-mandelate dehydrogenase

Mutants 123 and HMM5 were grown in 5mM-L-glutamate/salts P2 medium at 23°C and 30°C respectively (Methods 6.8 and 6.9). The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted (Methods 9.1.1). The protein contents of the extracts were determined (Methods 20) and the extracts were mixed in different proportions to give samples with different specific activities of D-mandelate dehydrogenase. The activity of each sample was determined six times with substrate and corrected by determining the activity twice without substrate. Points on the graph represent the mean activities and the vertical bars, the standard deviations. The line of best fit was calculated by the least squares regression method using all the activities calculated for each sample (correlation coefficient, r = 0.99)

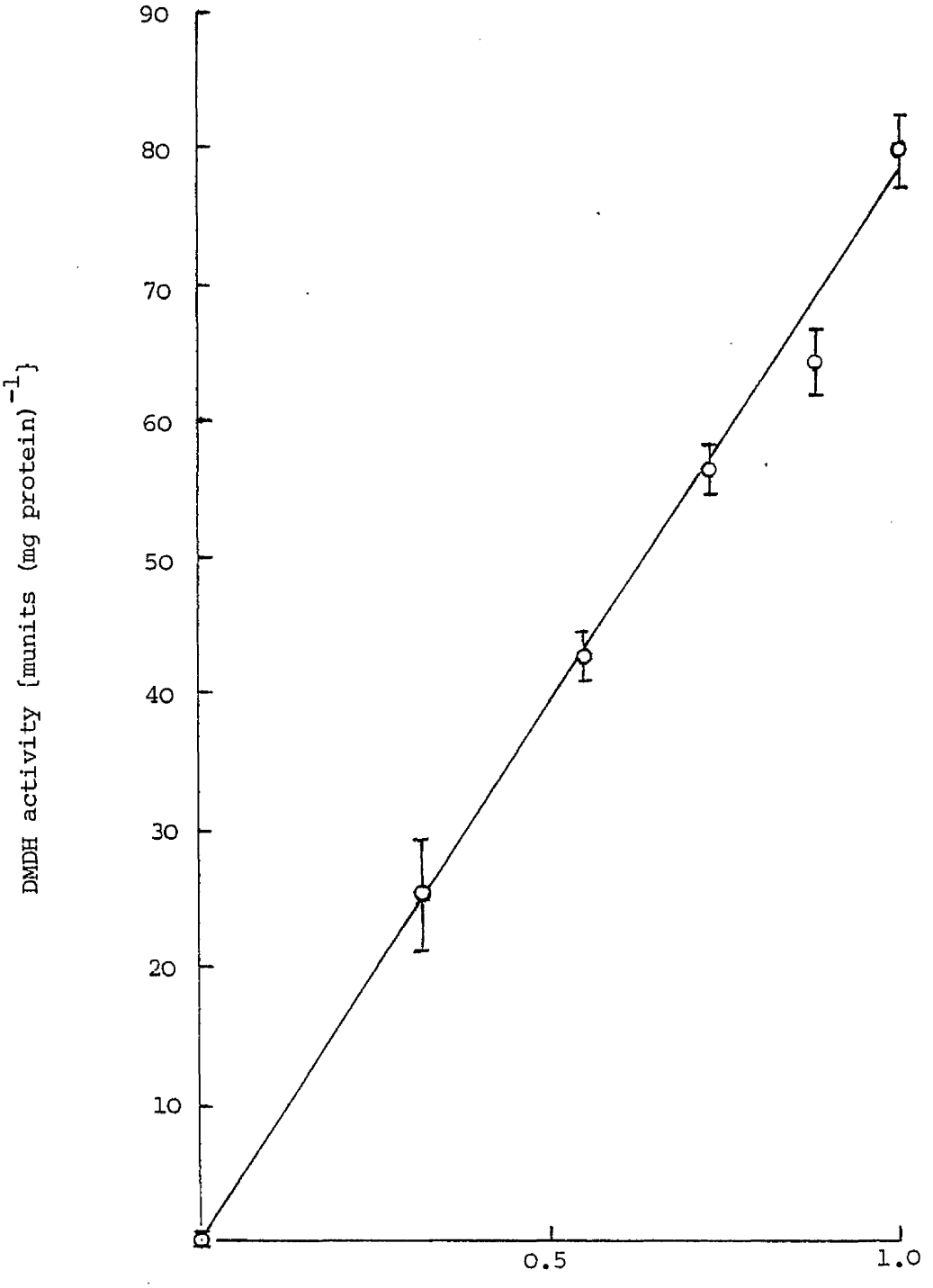
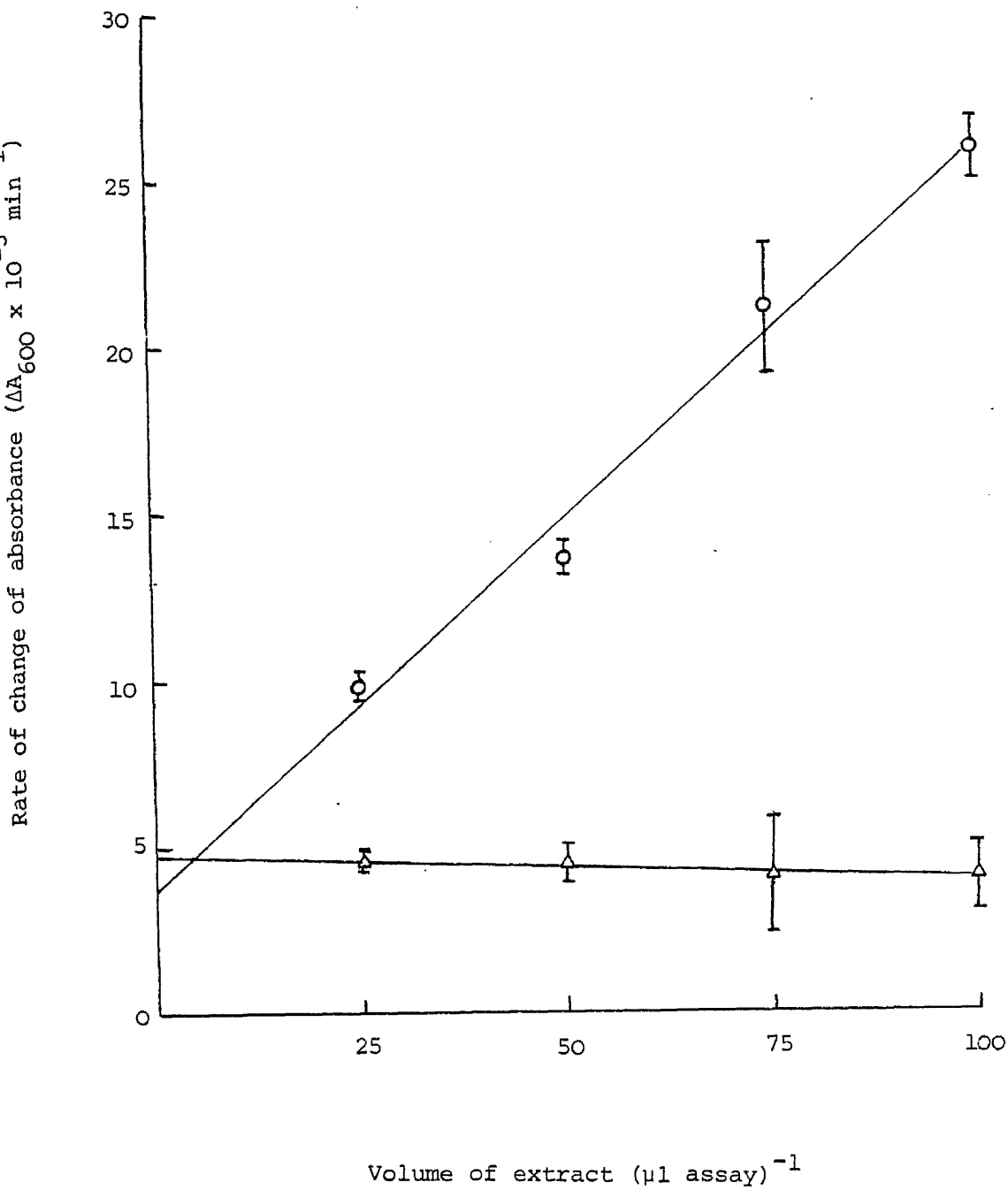

90
80
70
60
50
40
30
20
10
0.5
1.0
DMDH activity [munits (mg protein)$^{-1}$]
ratio of protein in the mixed extracts
(strain 123 : strain HMM5)

Fig. 10 Effect of extract volume on the measured D-mandelate dehydrogenase activity

Mutant 219 was grown at 23°C in 5mM-L-glutamate/salts P2 medium (Methods 6.8). The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted (Methods 9.1.1). D-Mandelate dehydrogenase activity was then measured using 25, 50, 75 or 100µl extract in a standard mixture (Methods 11.1). The activity was determined six times with substrate and twice without substrate. Points on the graph represent the mean activity, and the vertical bars the standard deviation. The lines of best fit were calculated by the least-squares linear regression method.

O : activity in the presence of substrate,
correlation coefficient = 0.99

Δ : activity in the absence of substrate
correlation coefficient = 0.92


Rate of change of absorbance (ΔA_{600} x 10^{-3} min^{-1})
30
25
20
15
10
5
0
25
50
75
100
Volume of extract (µl assay)$^{-1}$

3.5 Variations due to growing bacteria on different days

The reproducibility of the specific activity of D-mandelate dehydrogenase found in different batches of bacteria, grown on separate occasions, was deliberately tested with strain 219 because this strain is particularly susceptible to lysis (Results 1.1). Independent cultures were therefore grown from 72h and 48h nutrient broth inocula on five different days. Although the 72h inocula gave a higher mean activity than the 48h inocula, the activity of bacteria grown from each type of inoculum was reasonably constant (Table 9 a, b). This was despite the fact that some differences in the growth pattern were observed from day to day and lysis followed by regrowth (e.g. Fig. 2) sometimes occurred. It may be noted that the most extreme values (15.3.79) correspond to the batch cultures shown in Fig. 2.

3.6 Reproducibility of the disruption and assay procedures

3.6.1 Variations due to ultrasonic disruption

To determine if disruption procedures were reproducible, one bacterial suspension was divided into eight samples, and each sample then ultrasonically disrupted and assayed for D-mandelate dehydrogenase activity (Table 10 a). The low standard deviation of the summation of the means implies that the sonication procedures used are very reproducible.

3.6.2 Variations amongst assays

The low standard deviation (approximately 7% of the mean) of the mean (Table 10 b), both in the presence and absence of substrate indicates good reproducibility of the assay procedure.

Table 9 Variations associated with growing bacteria on different days

Mutant 219 was grown in 5mM-L-glutamate/salts P2 medium at 23°C from 72h and 48h nutrient broth inocula (Methods 6.8). The bacteria were harvested, washed and stored (Methods 7.1). This procedure was done on five days using independent inocula on each occasion. The bacteria were all ultrasonically disrupted on the same day (Methods 9.1.1) and the D-mandelate dehydrogenase activity measured (Methods 11.1).

Date	D-mandelate dehydrogenase activity munits (mg protein)$^{-1}$	
	mean	S.D. for triplicate assays
(a) 72h nutrient broth inocula		
13.3.79	153	2.3
14.3.79	131	8.7
15.3.79	160	6.5
16.3.79	119	2.1
19.3.79	147	3.7
mean for all five days	142 $\pm$	standard deviation of the mean 15.0
(b) 48h nutrient broth inocula		
13.3.79	109	6.1
14.3.79	123	1.9
15.3.79	103	1.1
16.3.79	110	2.1
19.3.79	132	2.0
mean for all five days	115 $\pm$	standard deviation of the mean 10.6

Table 10 Reproducibility of the disruption and assay procedures

(a) Variations due to sonication

Mutant 219 was grown at 23°C in 5mM-L-glutamate/salts P2 medium (Methods 6.8). The bacteria were harvested, washed and stored as described in Methods 7.1. The bacteria were resuspended to A_{500} = 5 in 0.1M-potassium phosphate buffer, pH7.5 and eight samples (5ml) ultrasonically disrupted using probe number 1 as described in Methods 9.1.1. After centrifugation each extract was assayed three times for D-mandelate dehydrogenase activity (Methods 11.1). The values are not corrected for endogenous activity.

(b) Variations amongst assays

Mutant 219 was grown and harvested as in (a). The bacteria were ultrasonically disrupted as described in Methods 9.1.1 using probe number 1. After centrifugation the extract was assayed for D-mandelate dehydrogenase activity (Methods 11.1), thirty-six times with extract and twelve times without.

(a)

Ultrasonically disrupted sample	D-mandelate dehydrogenase activity munits (mg protein)$^{-1}$	
	mean	S.D. for triplicate assays
1	42.9	0.6
2	46.5	0.2
3	40.4	0.2
4	44.0	1.9
5	46.6	0.7
6	39.6	3.2
7	48.4	0.3
8	45.1	0.1

summation of means 44.2 ± 2.8 (8)

(b)

D-mandelate in reaction mixture	D-mandelate dehydrogenase activity munits (mg protein)$^{-1}$		Number of determinations
	mean	S.D.	
+	44.8	2.6	36
-	12.6	0.9	12

3.7 Effects of freezing and storing on the activity of D-mandelate dehydrogenase

Since it was inconvenient to assay the enzyme on the same day as the bacteria were harvested, the bacteria were stored at -18°C until they were ultrasonically disrupted. The results in Table 11 show that there was practically no loss in enzyme activity on freezing or during storage.

These results also gave an indication of the total reproducibility of the disruption and assay procedures from day to day.

Table 11 Effects of freezing and storing on the activity of D-mandelate dehydrogenase

Two experiments were done on different occasions. In each case mutant 219 was grown in 5mM-L-glutamate/salts P2 medium at 23°C (Methods 6.8). The bacteria were harvested, washed and either stored at -18°C (Methods 7.1) or resuspended immediately for ultrasonic disruption (Methods 9.1.1). In the first experiment one sample was disrupted on each occasion and the extract assayed for D-mandelate dehydrogenase activity (Methods 11.1). In the second experiment three samples were disrupted on each occasion and assayed, and so each value quoted is the mean of nine determinations.

Storage time	D-mandelate dehydrogenase activity munits (mg protein)$^{-1}$	
Expt. 1	mean	standard deviation for three assays
none	103	2.1
1d	100	1.4
Expt. 2	mean	standard deviation for nine assays
none	126	3.1
1d	117	1.6
3d	128	2.7
5d	100	8.0
11d	110	5.8

summation of means = 112 $\pm$ standard deviation of the mean 11 (7)

4. Oxygen uptake

The effect of DCIP, PMS and BSA on oxygen consumption by both a membrane fraction and an extract of mutant 219 was examined using either D- or L-mandelate as substrate.

Table 12 shows the oxygen uptake measured using a membrane fraction; an example of one of the traces obtained in these experiments is given in Fig. 11. When either D- or L-mandelate were added as substrates (ii, Table 12) the rate of oxygen uptake was greatly enhanced above the endogenous rate (i), although the rate of oxygen consumption with D-mandelate was only half that obtained with L-mandelate.

When DCIP was added, oxygen uptake ceased for a short period whilst the dye was reduced (see Fig. 11 C to D, although this reaction mixture also contained BSA). This implied that the dye was reduced in preference to oxygen. After decolourization of the dye, oxygen consumption resumed although the rate was only half that with substrate alone (iii). Addition of PMS gave an approximately five-fold increase in oxygen consumption with either substrate (iv compared with ii), or with substrate and reduced DCIP (v compared with iii). Even with PMS, however, the rate of oxygen uptake in the presence of DCIP was still lower than that obtained without DCIP (v compared with iv). Subsequent addition of BSA to the reaction mixture containing PMS, and reduced DCIP restored the rate of oxygen consumption to about that obtained with PMS alone (vi). In contrast a much smaller increase in oxygen consumption was observed when BSA was added to substrate alone (vii compared with ii) or substrate + PMS (viii compared with iv). These results suggest that BSA relieves an inhibitory effect of reduced DCIP.

Table 12 Oxygen uptake by a membrane fraction of mutant 219 using D- and L-mandelate as substrates

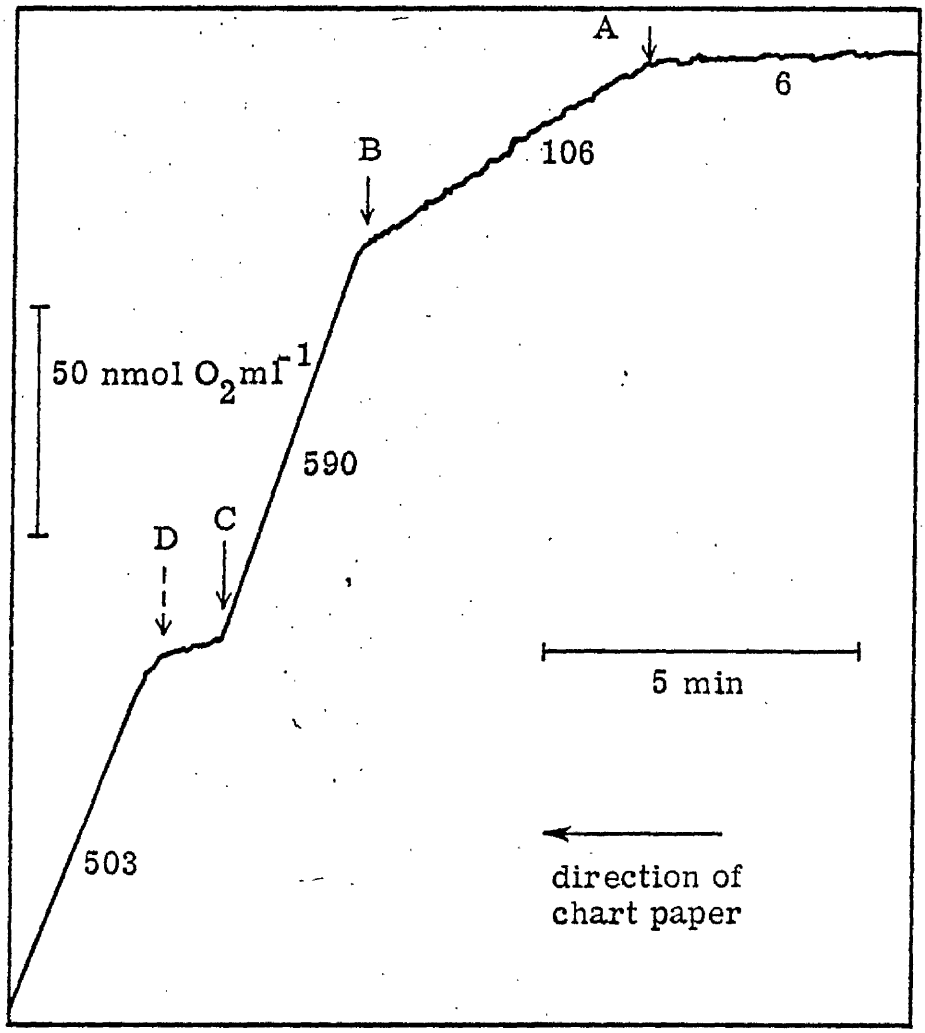
Mutant 219 was grown in 5mM-L-glutamate/salts P2 medium at 23°C as described in Methods 6.8. The bacteria were harvested, washed, stored and ultrasonically disrupted in a small Rosett cell, and a membrane fraction prepared (Methods 7.1 and 9.2). Oxygen uptake was measured as described in Methods 15, using 0.2ml of membrane fraction. The protein content of the membrane fraction was 5.9mg ml^{-1}.

	Additions	Oxygen uptake* nmol O_2 min^{-1} (ml extract)$^{-1}$	
		D-mandelate	L-mandelate
i	none	7 ± 6(9)	
ii	substrate	92 ± 16(4)	233 ± 8(5)
iii	substrate, DCIP	46(1)	129(1)
iv	substrate, PMS	502 ± 6(2)	1032 ± 34(2)
v	substrate, DCIP, PMS	257 ± 36(2)	710 ± 124(2)
vi	substrate, DCIP, PMS, BSA	450 ± 82(5)	1020 ± 28(3)
vii	substrate, BSA	120 ± 19(2)	256 ± 4(2)
viii	substrate, BSA, PMS	620 ± 29(3)	1228 ± 265(3)

*Rates are corrected by subtraction of the endogenous rate of oxygen uptake (i)

Fig. 11 Measurement of oxygen uptake with D-mandelate for a membrane fraction of mutant 219

Mutant 219 was grown in 5mM-L-glutamate/salts P2 medium at 23°C as described in Methods 6.8. The bacteria were harvested, washed, stored and ultrasonically disrupted in a small Rosett cell, and a membrane fraction prepared (Methods 7.1 and 9.2). At the start of the experiment the reaction mixture contained membrane fraction (0.2ml) and BSA (10mg), buffer and water to a final volume of 3.0ml. The endogenous rate of oxygen uptake was measured and the following additions were made through the hole in the perspex disc; A, 15µmol D-mandelate; B, 1µmol PMS; C, 200nmol DCIP. D indicates the time when the DCIP became colourless. The rates of oxygen consumption are quoted as nmol O_2 min^{-1} $(ml)^{-1}$. The protein content of the membrane fraction was 5.9mg ml^{-1}.


A
6
B
106
50 nmol O_2 ml^{-1}
590
D
C
5 min
503
direction of
chart paper

In a subsequent experiment an extract of 219 was found to give similar results to those obtained for the membrane preparation. In particular, addition of DCIP again caused a cessation of oxygen uptake until complete reduction of DCIP had occurred. The subsequent lowering of the rate of oxygen uptake could be partially relieved by addition of BSA. When BSA was present before DCIP addition, however, no initial cessation of oxygen uptake was observed. Instead there was a gradual decolourization of the dye with only a slight decrease in the rate of oxygen uptake. It appears therefore that prior addition of BSA prevents the preferential reduction of DCIP. When PMS was also present, added DCIP was rapidly reduced in preference to oxygen regardless of the presence of BSA (see also C to D in Fig. 11).

In the oxygen electrode, it can be assumed that oxidation of 1mol of mandelate required ½mol of O_2 if the end product was water, or 1mol of O_2 if hydrogen peroxide was formed. Since the inclusion of catalase (38,750units) in the reaction mixture was without effect (results not shown), it seems that water is the final product. It should therefore be possible to correlate rate of oxygen consumption with the rate of DCIP reduction observed spectrophotometrically. Using the rates of oxygen uptake measured in the presence of BSA, PMS and DCIP (Table 11 , line vi), expected enzyme activities of 900 and 2040nmol min^{-1} (ml extract)$^{-1}$ were calculated for D- and L-mandelate dehydrogenase respectively. The rates were measured spectrophotometrically on the same extracts and found to be 830 and 1994nmol min^{-1} (ml extract)$^{-1}$ respectively. Thus both sets of results are mutually consistent.

5. Comparison of the properties of D- and L-mandelate dehydrogenase

Whenever possible, comparative studies were carried out using mutant 219, which possesses both D- and L-mandelate dehydrogenase activities. In this way, the same extract could be used for studying both enzymes and so the differences between the two assays were minimised. In order to decrease further any possible artefactual differences in results obtained in measuring the two enzymes, it seemed wise to make the reaction mixtures as similar as possible. Since the original system for measuring L-mandelate dehydrogenase contained neither PMS nor BSA (Methods 11.2.1), the effects of including both compounds were examined since they were needed in the D-mandelate dehydrogenase assay.

Inclusion of PMS in the L-mandelate dehydrogenase assay gave some increase in activity (Fig. 12). At the concentration of PMS employed in the D-mandelate dehydrogenase assay (0.33mM) an increase of 35% was observed for L-mandelate dehydrogenase.

The effect of added BSA was then studied using 0.33mM PMS (Fig. 13). Activity initially increased as BSA concentration was increased, displaying a broad maximum over the range 0.13 - 0.67mg BSA ml^{-1}. At higher BSA concentrations the activity decreased until at 6.7mg ml^{-1} there was no stimulation, and at 13.3mg ml^{-1} the enzyme activity was inhibited by 38%.

The inclusion of 0.33µmol PMS and 3.33mg BSA per ml in the L-mandelate dehydrogenase assay (Methods 11.2.2), in other words the same conditions as used for D-mandelate dehydrogenase, gave an overall stimulation of about 20%.

Fig.12 The effect of PMS on the activity of L-mandelate dehydrogenase

Mutant HMM5 was grown in 40mM-L-glutamate/salts P2 medium supplemented with a mixture of trace metals at 30°C as described in Methods 6.10. The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted (Methods 9.1.1). L-Mandelate dehydrogenase activity was then measured in the extract. In addition to enzyme, the assay mixtures contained (total volume 3.0ml):

200μmol potassium phosphate buffer, pH7.0
200nmol DCIP
appropriate concentrations of PMS
1.5μmol L-mandelate (adjusted to pH7.0) to initiate the reaction

Fig. 13 The effect of BSA on the activity of L-mandelate dehydrogenase

Mutant HMM5 was grown in 5mM-L-glutamate/salts P2 medium at 30°C (Methods 6.9). The bacteria were harvested, washed, stored and ultrasonically disrupted as described in Methods 7.1 and 9.1.1. L-Mandelate dehydrogenase activity was then measured in the extract. In addition to enzyme, the assay mixtures contained (total volume 3.0ml):

200μmol potassium phosphate buffer, pH7.0
200nmol DCIP
1μmol PMS
appropriate concentrations of BSA (dissolved in 0.1M-potassium phosphate buffer, pH7.5)
1.5μmol L-mandelate (adjusted to pH7.0) to initiate the reaction

Fig. 12

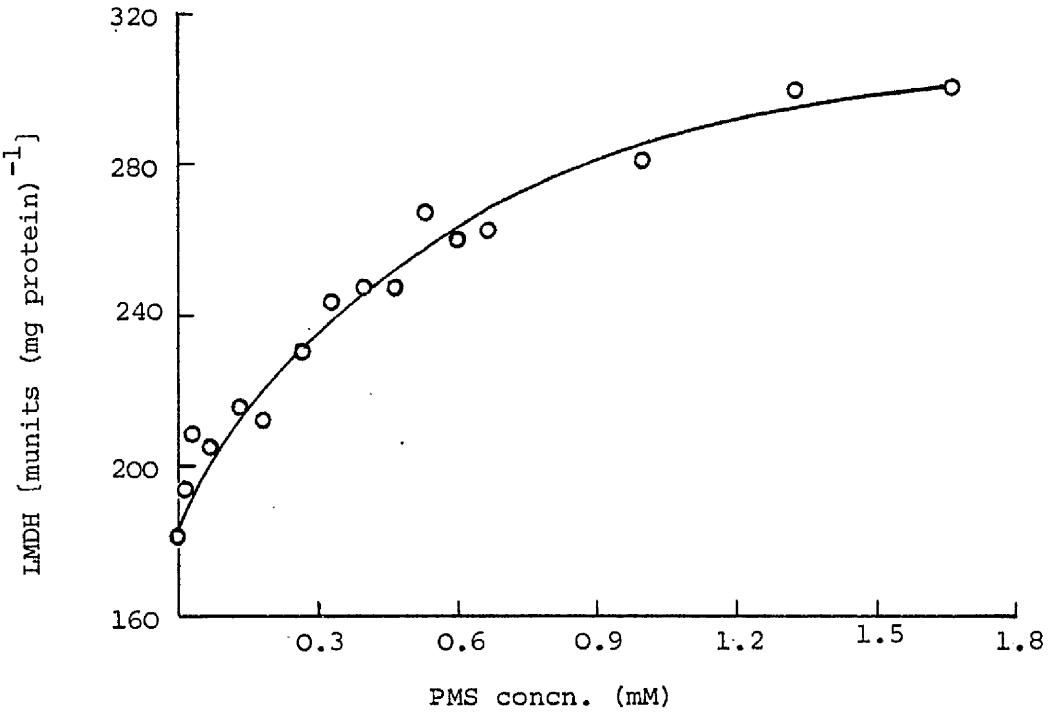
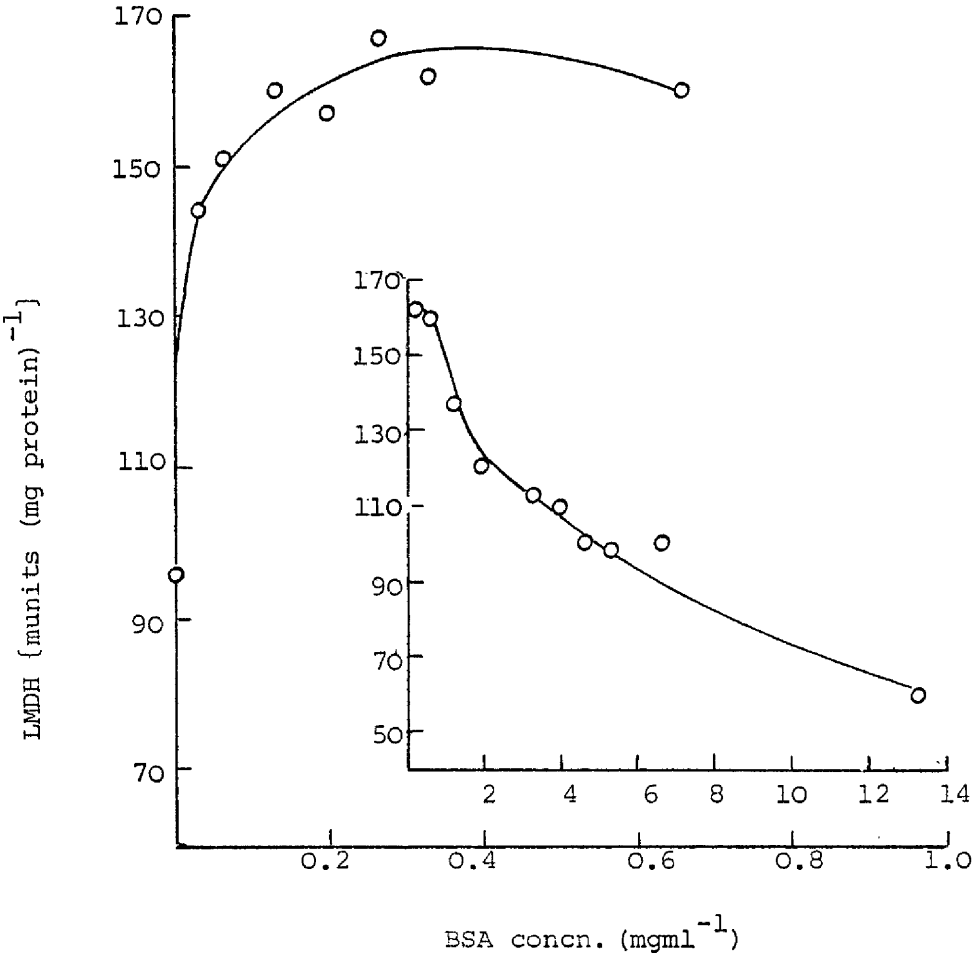

320
280
240
200
160
0.3
0.6
0.9
1.2
1.5
1.8
LMDH [munits (mg protein)$^{-1}$]
PMS concn. (mM)

Fig. 13


170
150
130
110
90
70
0.2
0.4
0.6
0.8
1.0
170
150
130
110
90
70
50
2
4
6
8
10
12
14
LMDH [munits (mg protein)$^{-1}$]
BSA concn. (mgml^{-1})

In all subsequent experiments described in this section in which the properties of the dehydrogenases are compared, PMS and BSA were included in both reaction mixtures and so the only differences between the two assays was that L-mandelate dehydrogenase was measured at pH7, and D-mandelate dehydrogenase at pH7.5.

A further advantage of adding BSA to the reaction mixture for the measurement of L-mandelate dehydrogenase was that the reaction proceeded linearly with respect to time, whereas in the absence of BSA the initial rate of reaction was always used to calculate activity (see p.107).

5.1 Determination of K_m and K_i for D- and L-mandelate dehydrogenase

Strains 123 and HMM5 were used as sources of D- and L-mandelate dehydrogenase activity respectively and results plotted as Lineweaver-Burk graphs (e.g. Fig. 14). Independent experiments gave a calculated K_m of 545 $\pm$ 262μM (4) for D-mandelate dehydrogenase, and a K_m of 207 $\pm$ 12μM (3) for L-mandelate dehydrogenase.

The activities of D- and L-mandelate dehydrogenases were also measured in the presence of L-mandelate (1 and 2.5mM) and D-mandelate (2.5 and 10mM) respectively. From appropriate Lineweaver-Burk plots for two experiments K_i values of 691 and 740μM were calculated for the inhibition of D-mandelate dehydrogenase activity by L-mandelate. The K_i value for the inhibition of L-mandelate dehydrogenase by D-mandelate was much higher at 4.08mM (result from Fig. 14).

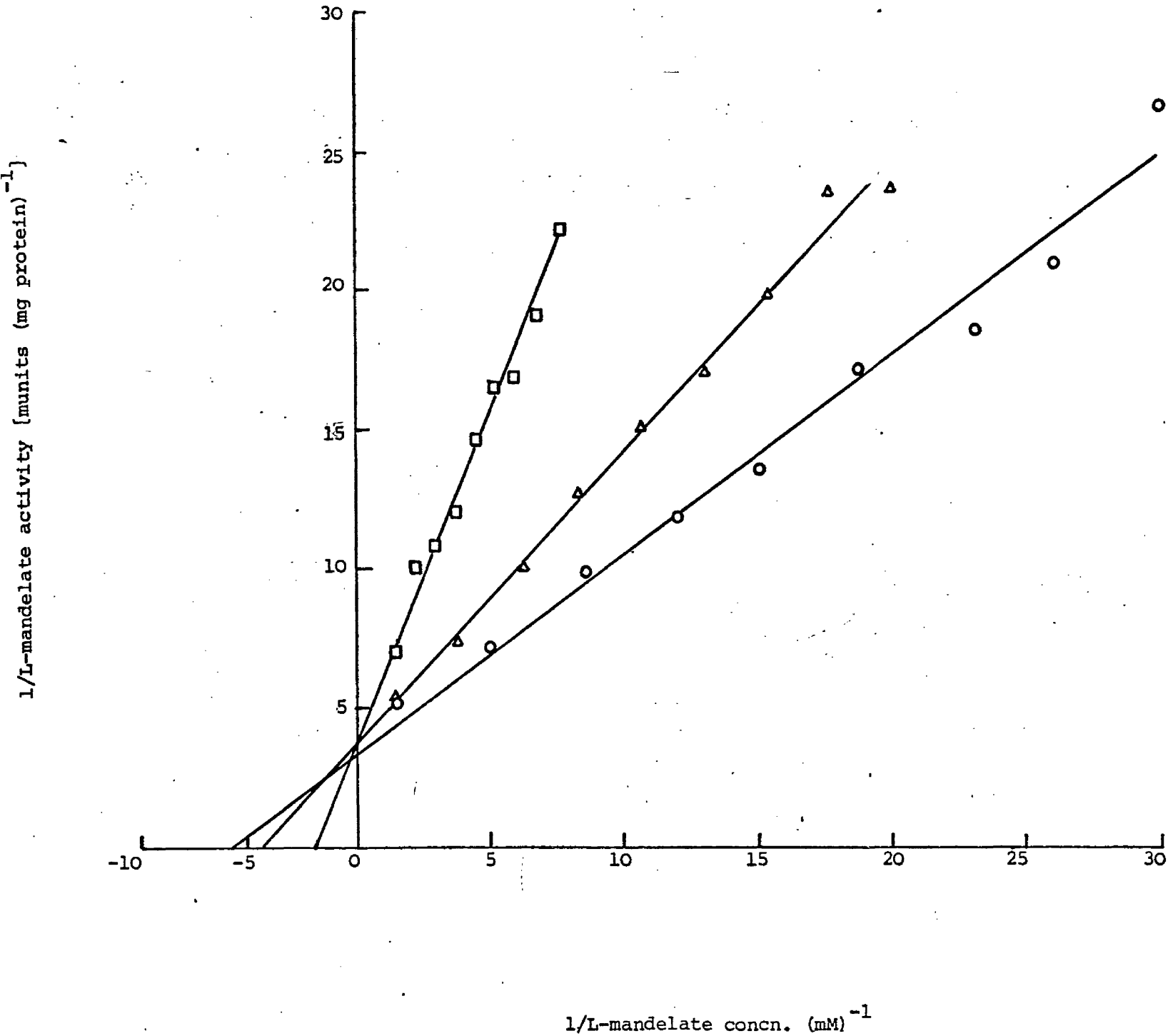
Fig. 14 Determination of K_m and K_i for L-mandelate dehydrogenase

Mutant HMM5 was grown at 30°C in 5mM-L-glutamate/salts P2 medium (Methods 6.9). The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted (Methods 9.1.1). The activity of L-mandelate dehydrogenase was measured as described in Methods 11.2.2 at nine different substrate concentrations in the presence and absence of the inhibitor D-mandelate. Each point represents one value corrected for endogenous activity. The lines of best fit were calculated by the least-squares linear regression method.

O : no inhibitor (correlation coefficient = 0.99)

Δ : 2.5mM-D-mandelate (final concn.; correlation coefficient = 0.99)

□ : 10mM-D-mandelate (final concn.; correlation coefficient = 0.99)


30
25
20
15
10
5
-10
-5
0
5
10
15
20
25
30
1/L-mandelate activity [munits (mg protein)$^{-1}$]
1/L-mandelate concn. (mM)$^{-1}$

5.2 Temperature dependence of the D- and L-mandelate dehydrogenase reactions

Fig. 15 shows the Arrhenius plots obtained by measuring the activity of D- and L-mandelate dehydrogenases over the temperature range 10-57°C. The plots are approximately linear over the range 10-25°C and the energies of activation were calculated using the Arrhenius equation:

$$d\log_{10}K = \frac{-E}{2.303R} \cdot d\left(\frac{1}{T}\right)$$

where E = energy of activation

R = gas constant $[8.314 JK^{-1}(mol)^{-1}]$

T = temperature K

For D-mandelate dehydrogenase the energy of activation was $42 kJmol^{-1}$, and for L-mandelate dehydrogenase $54 kJmol^{-1}$.

5.3 Effect of reagents which react with sulphydryl groups on the activities of D- and L-mandelate dehydrogenase

The results of pre-incubating extract for 10min with various thiol inhibitors are shown in Table 13. Of the five inhibitors tested, only iodoacetate failed to inhibit both enzymes and, indeed, seemed to give some activation at 0.1 and 1mM, although D-mandelate dehydrogenase was inhibited by about 25% at 10mM.

Both N-ethylmaleimide and iodoacetamide inhibited the two enzymes to comparable extents between 0.1 and 10mM, although relatively high concentrations were needed to give much inhibition.

The most convincing inhibition of activity was shown by mercuric chloride and p-chloromercuribenzoate. Both reagents inhibited D-mandelate dehydrogenase more strongly than L-mandelate dehydrogenase.

Fig. 15 Temperature dependence of the D- and L-mandelate dehydrogenase reactions

Mutant 219 was grown at 23°C in 5mM-L-glutamate/salts P2 medium (Methods 6.8). The bacteria were harvested, washed and stored (Methods 7.1) and then ultrasonically disrupted in a Rosett cell as described in Methods 9.1.2. The activity of D- and L-mandelate dehydrogenase was measured (Methods 11.1 and 11.2.2) at different temperatures over the range 10-57°C.

O : D-mandelate dehydrogenase

Δ : L-mandelate dehydrogenase

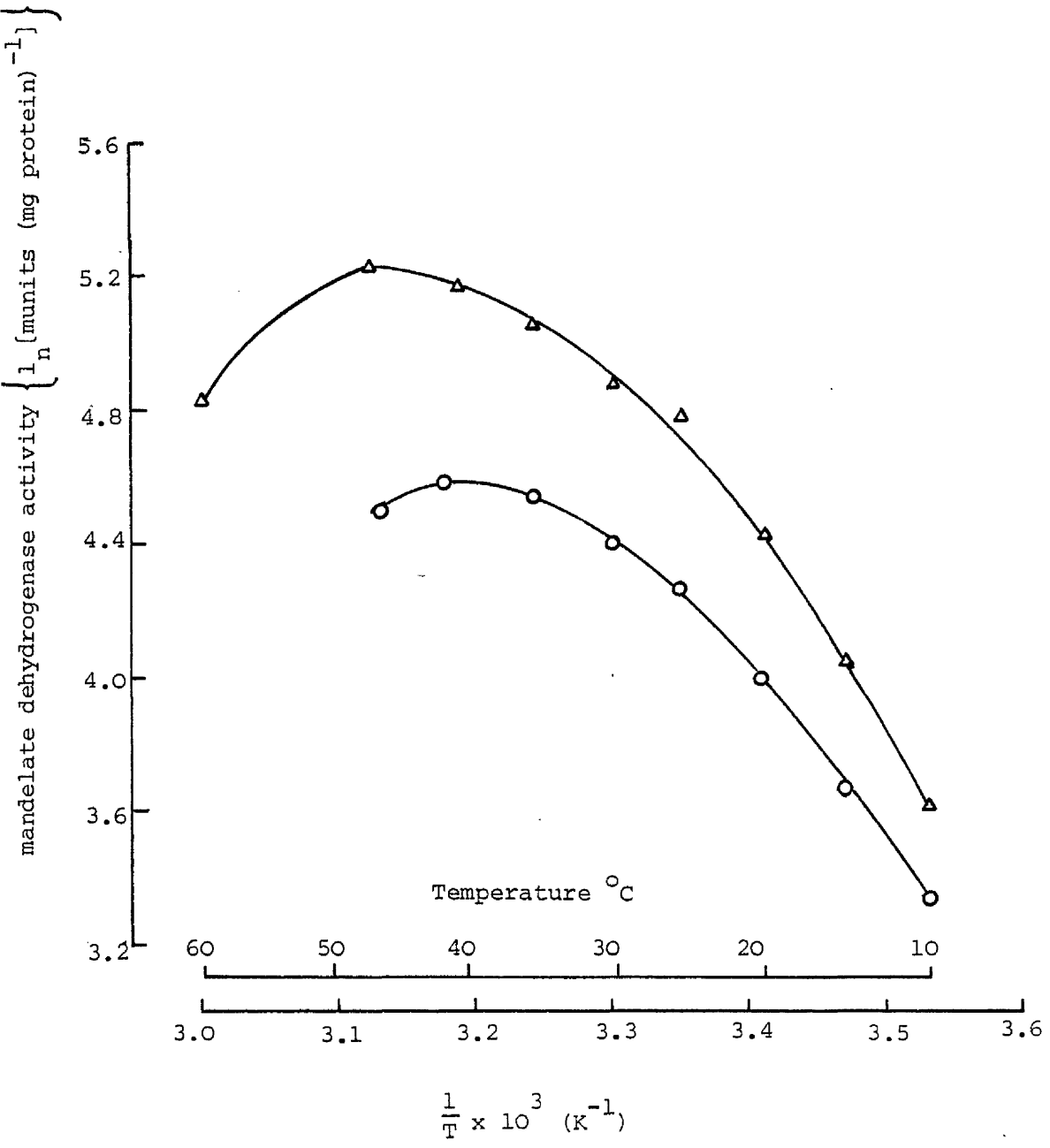

mandelate dehydrogenase activity {ln [munits (mg protein)$^{-1}$]}
5.6
5.2
4.8
4.4
4.0
3.6
3.2
Temperature °C
60
50
40
30
20
10
3.0
3.1
3.2
3.3
3.4
3.5
3.6
$\frac{1}{T} \times 10^3$ (K^{-1})

Table 13 Effect of reagents which react with sulphydryl groups on the activities of D- and L-mandelate dehydrogenase

Mutant 219 was grown in 5mM-L-glutamate/salts P2 medium (Methods 6.8). The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted in a Rosett cell as described in Methods 9.1.2. The inhibitors were pre-incubated with the extract and phosphate buffer (total volume 2.5ml) for 10min at 27°C before addition of the remaining assay components. The inhibitor concentration refers to the final concentration in the reaction. For each inhibitor three reaction mixtures were set up with a different concentration of inhibitor in each, and one reaction mixture without inhibitor. The activities of the four reaction mixtures were measured simultaneously and the activities in the presence of inhibitor were calculated as percentages of the activity obtained without inhibitor. The assays were done on different days and the uncorrected activities for D-mandelate dehydrogenase (Methods 11.1) were between 47 and 56 munits (mg protein)$^{-1}$ and for L-mandelate dehydrogenase (Methods 11.2.2) 104 to 124 munits (mg protein)$^{-1}$.

Inhibitor	Inhibitor concentration (mM)	Activity (% activity obtained without inhibitor)	
		DMDH	LMDH
N-Ethylmaleimide	0.1	94	98
	1	85	79
	10	21	23
Iodoacetamide	0.1	97	97
	1	79	90
	10	5	12
Iodoacetate	0.1	111	109
	1	124	102
	10	75	103
Mercuric chloride	0.1×10^{-3}	105	103
	1×10^{-3}	31	85
	10×10^{-3}	14	34
p-Chloromercuri-benzoate	0.7×10^{-3}	38	104
	10×10^{-3}	22	72
	300×10^{-3}	10	53

This effect was especially pronounced with p-chloromercuribenzoate; in a series of similar experiments to those shown in Table 13 it was found that about four hundred-fold higher concentrations of p-chloromercuribenzoate were required to obtain inhibition of L-mandelate dehydrogenase comparable to that of D-mandelate dehydrogenase. This compared with $HgCl_2$ where the concentration required for inhibition of L- mandelate dehydrogenase was only about four to ten-fold higher than that required for comparable inhibition of D-mandelate dehydrogenase.

A similar degree of inhibition of D-mandelate dehydrogenase was obtained with both reagents; for example, 0.7μM p-chloromercuribenzoate inhibited activity by about 62%, and 1μM $HgCl_2$ inhibited activity by about 69%. There were, however, considerable differences between the two compounds in the inhibition of L-mandelate dehydrogenase. For this enzyme, $HgCl_2$ seems a more effective inhibitor as about 66% inhibition was achieved with 10μM $HgCl_2$, whilst only about 47% inhibition was obtained with 300μM p-chloromercuribenzoate.

5.4 Effect of metal chelating reagents and related compounds on the activities of D- and L-mandelate dehydrogenase

EDTA had virtually no effect on either enzyme activity (Table 14) even at 10mM. Only o-phenanthroline and 8-hydroxyquinoline gave much inhibition of activity at 1mM. At the relatively high concentration of 10mM, 2,2-bipyridyl, KCN and NaN_3 inhibited in some cases. None of these compounds inhibited very strongly and in some cases there was slight stimulation of one or both of the enzyme activities.

In contrast oxalic acid inhibits both enzymes strongly and to the same extent, causing about 50% inhibition at 0.025mM.

Table 14 Effect of metal chelating reagents and related compounds on the activities of D- and L-mandelate dehydrogenase

Mutant 219 was grown in 5mM-L-glutamate/salts P2 medium (Methods 6.8). The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted in a Rosett cell as described in Methods 9.1.2. The potential inhibitors were pre-incubated with the extract and phosphate buffer (total volume 2.5ml) for 10min at 27°C before addition of the remaining assay components. The inhibitor concentration refers to the final concentration in the reaction. For each inhibitor three reaction mixtures were set up with a different concentration of inhibitor in each, and one without inhibitor. The activities of the four reaction mixtures were measured simultaneously, and the activities in the presence of inhibitor were calculated as percentages of the activity obtained without inhibitor. The assays were done on different days and the uncorrected activities for D-mandelate dehydrogenase (Methods 11.1) were between 37 and 52 munits (mg protein)$^{-1}$, and for L-mandelate dehydrogenase (Methods 11.2.2) 97 and 104 munits (mg protein)$^{-1}$.

Potential Inhibitor	Inhibitor concentration (mM)	Activity (% activity obtained without added reagent)	
		DMDH	LMDH
EDTA	0.1	101	104
	1	100	103
	10	101	109
o-Phenanthroline	0.01	99	100
	0.1	98	92
	1	87	79
8-Hydroxyquinoline	0.01	107	89
	0.1	104	95
	1	48	99
2,2-Bipyridyl	0.1	100	107
	1	102	106
	10	100	73
KCN	0.1	105	108
	1	102	109
	10	43	121
NaN_3	0.1	104	104
	1	115	114
	10	62	116
Oxalic acid	0.005	74	77
	0.025	50	48
	0.5	21	14

5.5 Effect of pH on the activity of D- and L-mandelate dehydrogenase

The assay pH was varied over the range pH5.7 to 9.5 by adjusting the pH value of the phosphate buffer and, if necessary, the pH of the substrate (Fig. 16). Potassium phosphate buffer (0.1M) was used for the entire range; this was done to avoid possible complications which might have been introduced by altering the buffering agent. It is realized that phosphate buffer gives different ionic strengths at different pH values and is a poor buffer at the extreme values tested; however, so far as the latter point is concerned, the actual pH values of the reaction mixture were recorded and are plotted in Fig. 16. Activities of both D- and L-mandelate dehydrogenases showed maxima at about pH7.8. L-mandelate dehydrogenase, however, appears to be less sensitive to pH, having greater than half maximal activity between pH6.0 and 9.0, whereas the comparable range of D-mandelate dehydrogenase is only pH6.8 to 8.5.

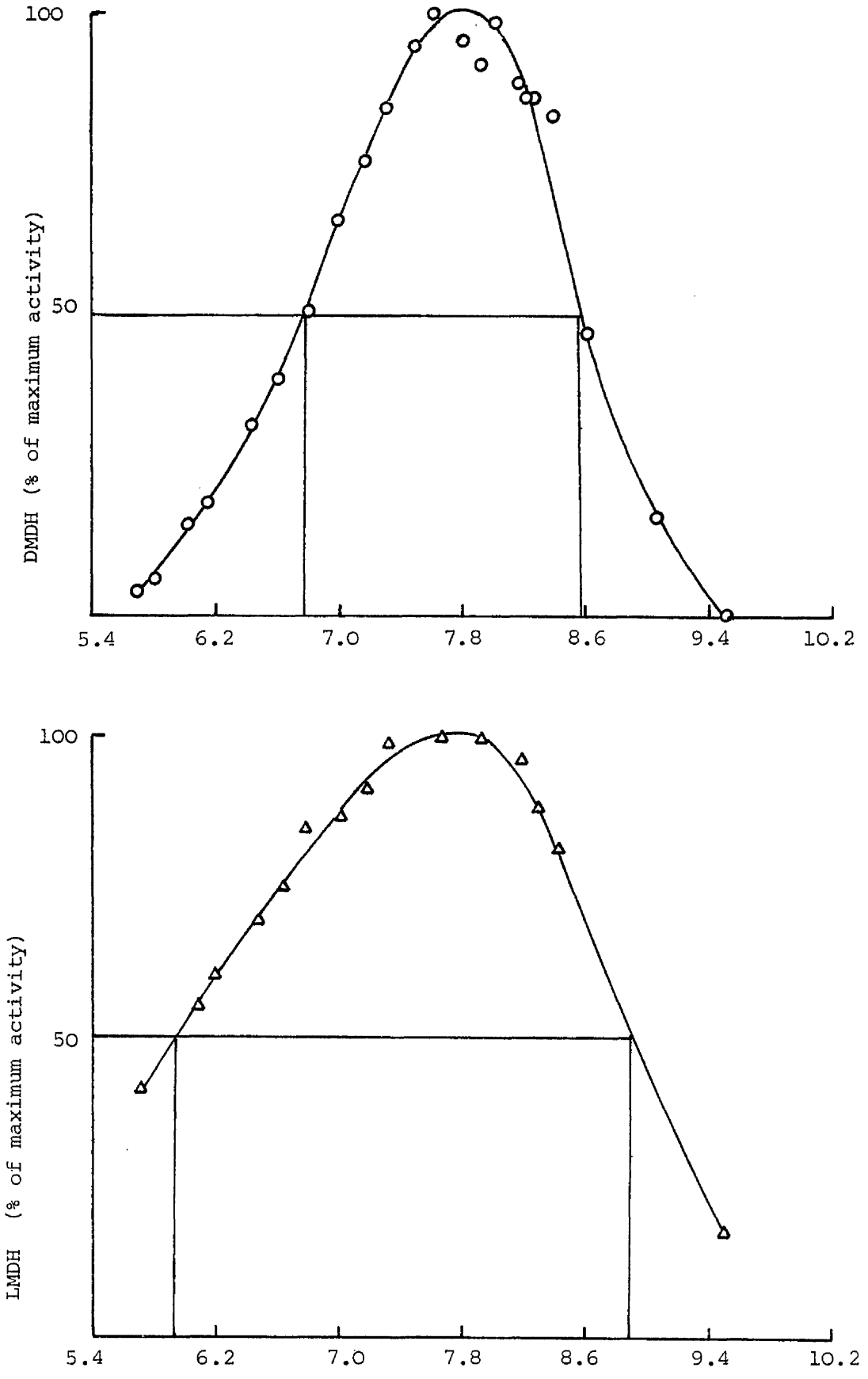
Inclusion of PMS and BSA appears slightly to increase the optimal pH of L-mandelate dehydrogenase from pH7.0 (Kennedy, 1967) to 7.8.

Fig. 16 Effect of pH on the activity of D- and L-mandelate dehydrogenase

Mutant 219 was grown at 23°C in 5mM-L-glutamate/salts P2 medium (Methods 6.8). The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted in a Rosett cell as described in Methods 9.1.2. The activities of D- and L-mandelate dehydrogenase were measured (Methods 11.1 and 11.2.2) using 0.1M-potassium phosphate buffer adjusted to different pH values. The pH values plotted in the graphs are the values of reaction mixtures measured after the reactions were complete. The activity of each enzyme is expressed as a percentage of the maximum activity [83 munits (mg protein)$^{-1}$ for D-mandelate dehydrogenase and 139 munits (mg protein)$^{-1}$ for L-mandelate dehydrogenase].

O : D-mandelate dehydrogenase

Δ : L-mandelate dehydrogenase


100
50
DMDH (% of maximum activity)
5.4
6.2
7.0
7.8
8.6
9.4
10.2
100
50
LMDH (% of maximum activity)
5.4
6.2
7.0
7.8
8.6
9.4
10.2
pH of reaction mixture

6. The regulation of D-mandelate dehydrogenase in mutants of *A. calcoaceticus* NCIB8250

The first three enzymes for L-mandelate metabolism in wild-type strain NCIB8250 are co-ordinately controlled (Livingstone & Fewson, 1972). Since D-mandelate is converted to phenylglyoxylate, and then appears to follow the same pathway as for the metabolism of L-mandelate it was obviously important to study the regulation of the new pathway. Two approaches were used:

(a) the induction, repression and anti-induction of the evolved D-mandelate dehydrogenase in strain 41 ($L^iD^iP^i$; Section 6.1).

(b) the expression of the evolved D-mandelate dehydrogenase in three classes of mutants which constitutively synthesize phenylglyoxylate carboxy-lyase (Section 6.2.2). The isolation of two of these classes of mutants is described here (Section 6.2.1), the third class having been isolated previously by Dr Fewson (unpublished work; Table 1).

6.1 Control of D-mandelate dehydrogenase in strain 41 under conditions of induction, repression and anti-induction

The appearance of D-mandelate dehydrogenase activity was compared with that of L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase under a range of conditions (Table 15).

When strain 41 was grown on L-glutamate alone no activity was observed showing that D-mandelate dehydrogenase is not synthesized consistutively in this strain.

Addition of L-mandelate (Table 15, 2) to the growth medium induced both L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase as expected, and also induced the evolved D-mandelate dehydro-genase. Similarly induction of all three enzymes was observed in the

Table 15 Induction of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase in mutant 41 under a range of conditions

Mutant 41 was grown at 23°C in 5mM-L-glutamate/salts P2 medium (Methods 6.8) containing:

1. No additions
2. 5mM-L-mandelate
3. 5mM-D-mandelate
4. 5mM-phenylglyoxylate
5. 5mM-phenylglyoxylate + 2.5mM-2-phenylpropionate
6. 2mM-thiophenoxyacetate
7. 5mM-phenylglyoxylate + 5mM-succinate

The bacteria were harvested when the turbidity reached A_{500} = 0.3 when grown in medium containing 2-phenylpropionate, and at A_{500} = 0.5-0.6 in all other media. The bacteria were washed, stored (Methods 7.1) and ultrasonically disrupted (Methods 9.1.1). Activities of D- and L-mandelate dehydrogenase were assayed in extracts (Methods 11.1 and 11.2.1) and phenylglyoxylate carboxy-lyase was assayed in toluenised bacterial suspensions (Methods 10 and 11.3). All assays were performed in triplicate, and when presented, the two values represent results for duplicate cultures.

Addition	Enzyme activity (munits (mg protein)$^{-1}$)		
	DMDH	LMDH	PC
1	1	0	0
	0	0	0
2	54	57	466
	48	58	395
3	46	52	541
	58	79	375
4	46	71	595
	69	96	629
5	0	0	0
6	14	24	109
	17	37	95
7	21	16	272
	19	20	175

presence of either D-mandelate (3), phenylglyoxylate (4), or thiophenoxyacetate (6). In the presence of the anti-inducer 2-phenylpropionate (5) no enzyme activity was measured.

The expression of the enzymes was also examined under conditions of catabolite repression by succinate (7). All three enzymes were co-incidently repressed.

The results of this work suggest that the evolved D-mandelate dehydrogenase behaves as a R_1 regulon enzyme. To firmly establish this, however, co-ordinate induction of D-mandelate dehydrogenase with the other R_1 regulon enzymes had to be demonstrated. Co-ordinacy between two enzymes requires that they be present in a constant ratio under various conditions. Thus co-ordinacy may be demonstrated if plotting the activity of one enzyme versus that of the other yields a straight line.

The results from Table 15 have been expressed as such plots in Fig. 17. The calculated correlation coefficients of the best straight lines show reasonably good correlation between D- and L-mandelate dehydrogenase (correlation coefficient 0.97) and between D-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase (correlation coefficient 0.94). The correlation between L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase (correlation coefficient 0.90) provides an internal control since these two enzymes are known to be co-ordinate in the wild-type strain NCIB8250 (Livingstone & Fewson, 1972). On this basis the correlation between D-mandelate dehydrogenase and the other two enzymes is good, suggesting that D-mandelate dehydrogenase is co-ordinately expressed with the R_1 regulon enzymes.

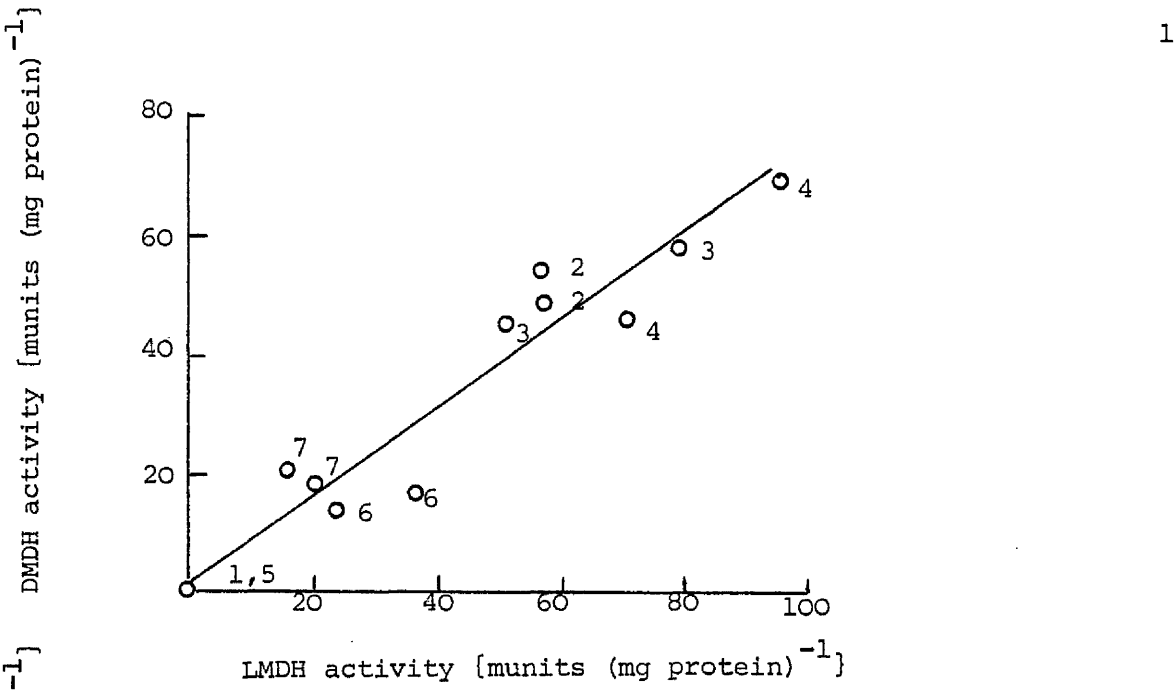
Fig. 17 Correlation of induction of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase in mutant 41 under a range of conditions

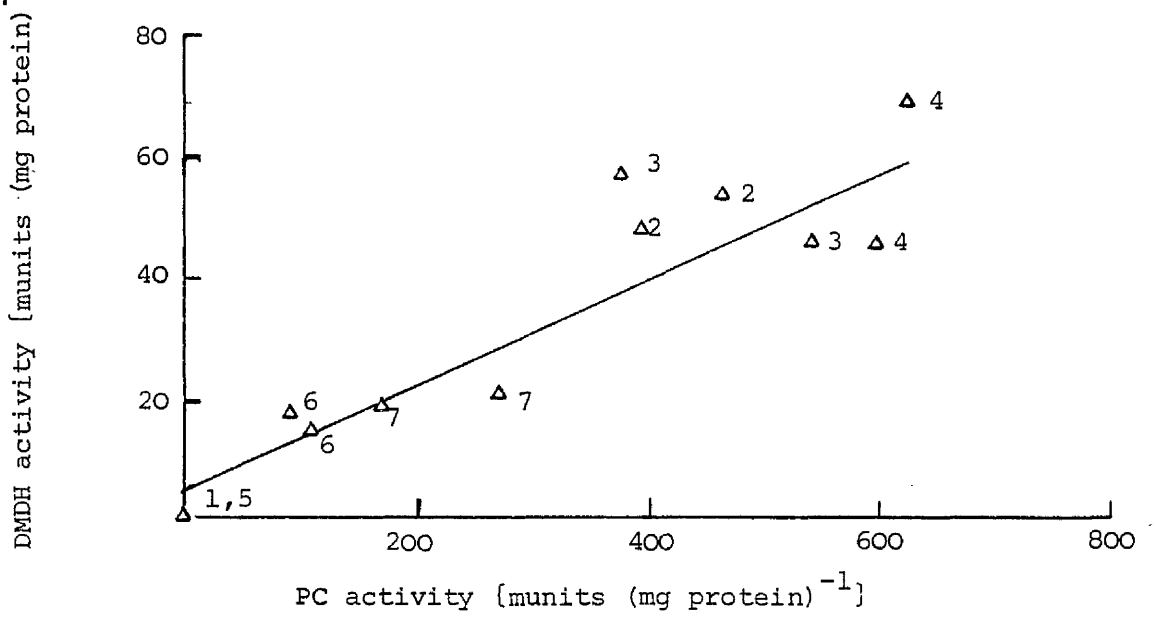
See legend to Table 15. The enzyme activities shown in Table 15 were plotted against each other and the lines of best fit calculated by the least squares linear-regression method.

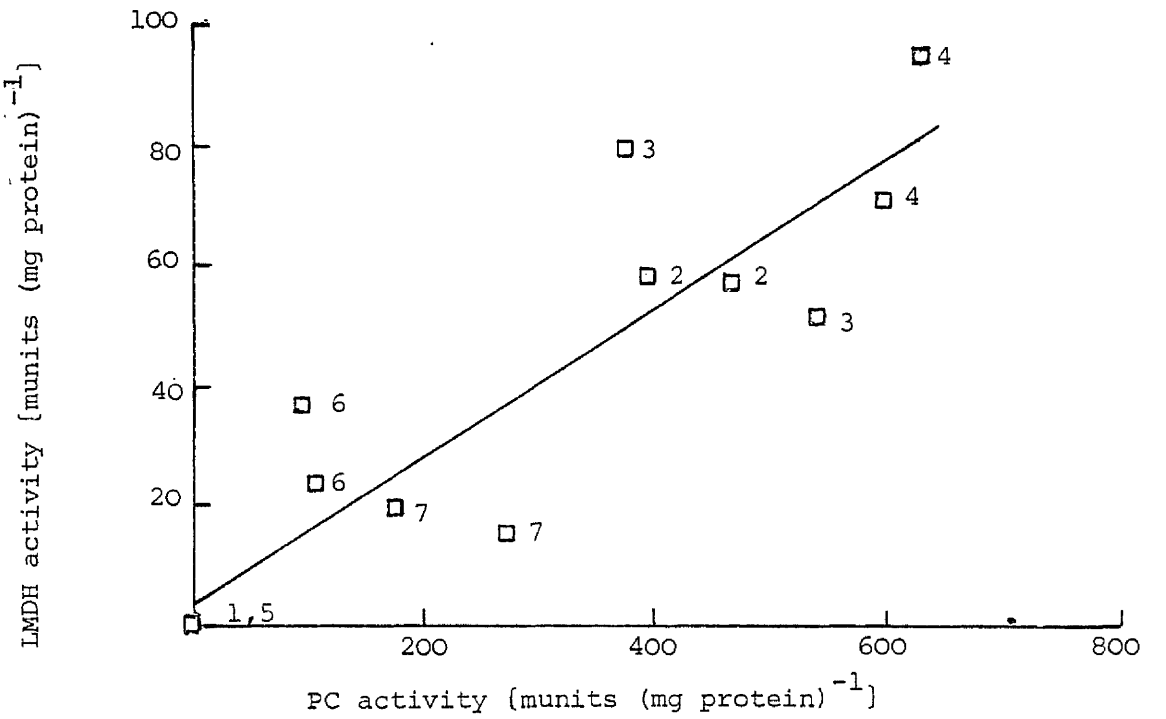
O : correlation coefficient = 0.97

Δ : correlation coefficient = 0.94

□ : correlation coefficient = 0.90


DMDH activity (munits (mg protein)$^{-1}$)
80
60
40
20
1,5
20
40
60
80
100
LMDH activity (munits (mg protein)$^{-1}$)


DMDH activity (munits (mg protein)$^{-1}$)
80
60
40
20
1,5
200
400
600
800
PC activity (munits (mg protein)$^{-1}$)


LMDH activity (munits (mg protein)$^{-1}$)
100
80
60
40
20
1,5
200
400
600
800
PC activity (munits (mg protein)$^{-1}$)

6.2 Isolation of constitutive mutants from strains D4OE and 41

Mutants able to synthesize phenylglyoxylate carboxy-lyase constitutively were isolated from strains D4OE ($L^{o}D^{i}P^{i}$) and 41 ($L^{i}D^{i}P^{i}$). Mutants of these strains able to grow on phenylglyoxylate in the presence of 2-phenylpropionate, an anti-inducer of the R_1 regulon enzymes (Fewson & Foote, 1976; Fewson et al., 1978), were isolated as the first step. However, although mutants constitutive for phenylglyoxylate carboxy-lyase would be expected to grow under such conditions, other classes of mutants might also be isolated e.g. those in which 2-phenylpropionate is no longer an anti-inducer. To identify those mutants constitutive for phenylglyoxylate carboxy-lyase, a screening procedure was developed which applied the observations of Beggs & Fewson (1977) that mutants constitutive for the R_1 regulon enzymes show poor growth on benzyl alcohol. The basis of this effect seems to involve repression of benzyl alcohol dehydrogenase activity by either phenylglyoxylate carboxy-lyase or possibly something co-ordinately expressed with it.

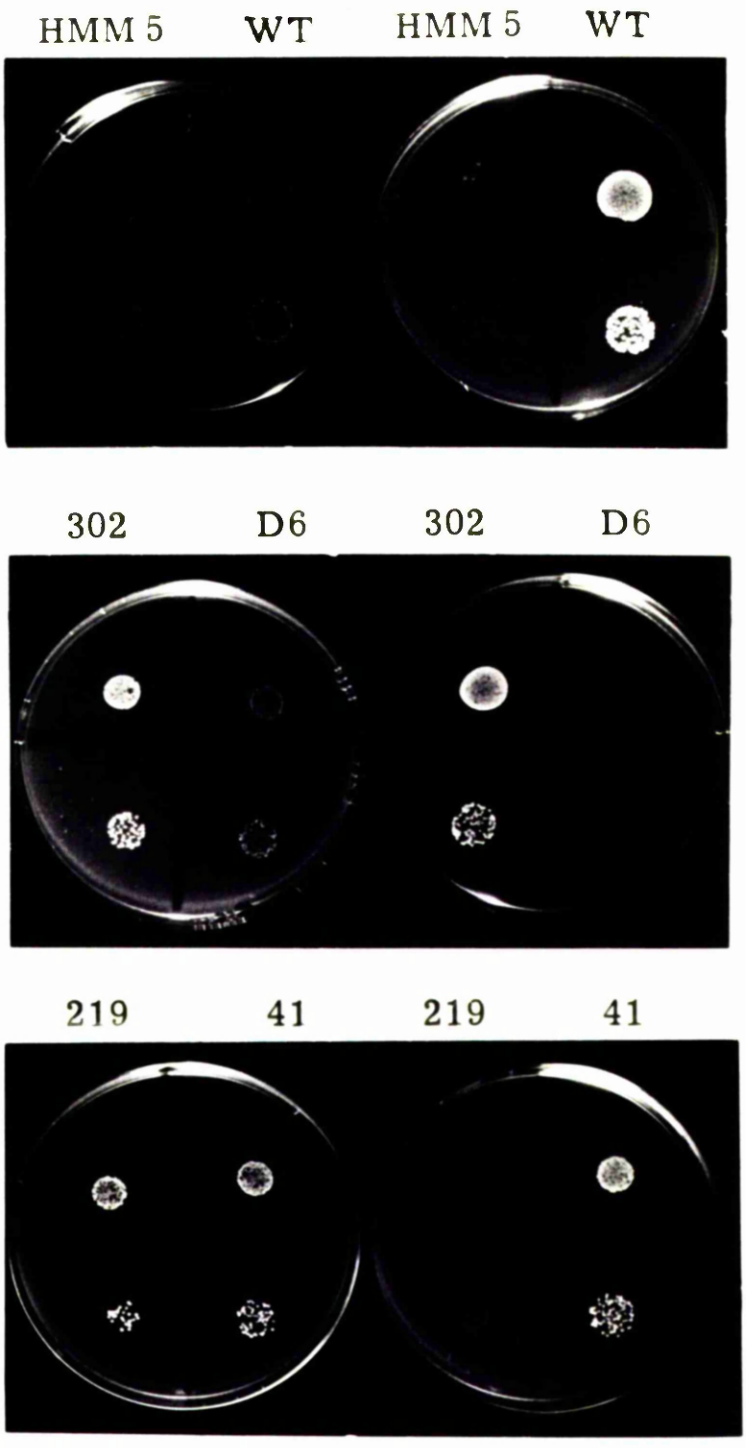
The screening procedure developed (Methods 8.1) involved growth of mutants and known control strains on benzyl alcohol/agar plates and used colony size as an indication of growth. Mutants were also grown on nutrient agar plates as a check for general viability.

Fig. 18 illustrates the effects observed when a number of mutants were grown on benzyl alcohol/salts agar or phenylglyxoylate/salts agar. It can be seen that wild-type strain NCIB8250, as well as mutants 302 and 41 which have inducible phenylglyoxylate carboxy-lyase, grow well on both types of plate. Mutants HMM5, D6 and 219 which have constitutive phenylglyoxylate carboxy-lyase however, only grow well on phenylglyoxylate.

Fig. 18 Growth of wild-type strain NCIB8250 and mutants on solid media containing phenylglyoxylate or benzyl alcohol as carbon source

Presumptive mutants and control strains were grown in nutrient broth (50ml) for 24h at 30°C. Serial dilutions were made in basal medium and one drop of a 10^{-4} and 10^{-5} dilution placed on plates (9cm diameter) containing 5mM-phenylglyoxylate/salts agar or 5mM-benzyl alcohol/salts agar. Dilutions of two mutants were placed on each plate and the plates were incubated for 48h at 30°C. In the photographs the top circle of growth in each case arose from the 10^{-4} dilution and the bottom circle from the 10^{-5} dilution of the nutrient broth culture.

WT : wild-type strain NCIB8250


HMM 5
WT
HMM 5
WT
302
D6
302
D6
219
41
219
41
Phenyglyoxylate
Benzyl Alcohol

6.2.1 Frequency of appearance of strains D4OE and 41 becoming constitutive for phenylglyoxylate carboxy-lyase

Spontaneous and NTG-induced mutants were isolated as described in Methods 8.2. The frequency of mutation was estimated using the following expression:

$$\text{Frequency of mutation} = \frac{\text{Mutants constitutive for PC}}{\text{Total number of bacteria spread}} \times \frac{\text{Number of colonies appearing}}{\text{Number of strains cloned}}$$

A frequency of spontaneous mutation of 9×10^{-8} was obtained for both strains (Table 16). The mutagen NTG caused a three-fold increase in mutation rate in strain D4OE, and a ten-fold increase in strain 41.

6.2.2 Enzyme activities measured in mutants derived from strains 41, D4OE and HMM5 which constitutively synthesize phenylglyoxylate carboxy-lyase

The regulation of D-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and, when appropriate, L-mandelate dehydrogenase was studied in the constitutive mutants isolated from strains D4OE and 41. In addition the mutants of HMM5 able to grow on D-mandelate (Fewson, unpublished work; Table 1 , Methods 2.1) were included in this study.

Table 17 a, b and c shows the enzyme activities of the three classes of mutant when grown in the absence of inducer; the parent strain was included for each class as a control.

All mutants constitutively synthesized D-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase when grown on the non-inducing carbon source L-glutamate. Under these conditions mutants derived from 41 (Table 17b) and HMM5(c) also constitutively synthesized L-mandelate dehydrogenase.

Table 16 The isolation of mutants of strains D4OE and 41 which constitutively synthesize phenylglyoxylate carboxy-lyase

Nutrient broth cultures of strain D4OE or 41 which had been grown for 24h at 23°C were spread on plates containing 2mM-2-phenylpropionate + 2mM-phenylglyoxylate/salts agar. Approximately 10^7 bacteria were spread on each plate. A few crystals (1-5) of NTG were put on some of the plates. Presumptive mutant colonies appeared during 10d incubation at 30°C. Several colonies were selected, cloned on 2mM-phenylpropionate + 2mM-phenylglyoxylate/salts agar and then tested for constitutive synthesis of phenylglyoxylate carboxy-lyase.

An estimate of the incidence of mutants was then made using the equation described in the text.

Parental strain	Mutagen	Total number of bacteria spread	Number of colonies appearing	Number of strains cloned	Mutants constitutive for PC	Calculated frequency of occurrence of mutants
D40E	NTG	5×10^{7}	295	39	2	3×10^{-7}
	None	1.5×10^{8}	307	67	3	9×10^{-8}
41	NTG	4×10^{7}	242	35	8	1×10^{-6}
	None	1.6×10^{8}	245	83	5	9×10^{-8}

Table 17 Activities of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase in mutants derived from strains 41(a), D4OE(b) and HMM5(c)

The mutants and parent strains were grown in 5mM-L-glutamate/salts P2 medium at 23°C as described in Methods 6.6. The bacteria were harvested, washed, stored and ultrasonically disrupted (Methods 7.1 and 9.1.1). The activities of D- and L-mandelate dehydrogenase were assayed in extracts (Methods 11.1 and 11.2.1). Phenylglyoxylate carboxy-lyase was assayed in duplicate using toluenised bacterial suspensions as described in Methods 10 and 11.3.

(a) Mutants derived from strain 41

Mutant	Enzyme activity (munits (mg protein)$^{-1}$)		
	DMDH	LMDH	PC
41	0	0	0
216	94	110	427
219	108	82	670
221	87	60	602
225	58	66	254
228	103	112	425
236	106	100	457
267	70	97	298
288	85	77	653
301	66	85	315
313	45	41	693
326	47	34	722
327	65	76	735

(b) Mutants derived from strain D4OE

Mutant	Enzyme activity (munits (mg protein)$^{-1}$)		
	DMDH	LMDH	PC
D4OE	0	0	0
123	119	0	578
129	114	0	688
148	20	0	166
157	62	0	334
165	67	0	500

(c) Mutants derived from strain HMM5

Mutant	Enzyme activity (munits (mg protein)$^{-1}$)		
	DMDH	LMDH	PC
HMM5	0	142	480
D3	42	39	187
D5	20	13	61
D6	79	111	579
D7	67	56	384

The results show that in every case the evolved D-mandelate dehydrogenase is co-incidently expressed with the original R_1 regulon enzyme(s). In this respect, the isolation criteria deserve comment. Mutants of strain D4OE ($L^{o}D^{i}P^{i}$) were isolated solely on the basis of possessing a constitutive phenylglyoxylate carboxy-lyase; there was no selective pressure for the mandelate dehydrogenase since the carbon source in the enrichment medium was phenylglyoxylate. However, under these conditions only mutants also constitutive for D-mandelate dehydrogenase were obtained. Similarly mutants of strain 41 ($L^{i}D^{i}P^{i}$) were isolated for constitutive synthesis of phenylglyoxylate carboxy-lyase, without any selective pressure on either dehydrogenase, yet both D- and L-mandelate dehydrogenases are also constitutively synthesized. The third class of mutants were isolated by a different route. The parent strain, HMM5, constitutively synthesizes phenylglyoxylate carboxy-lyase and L-mandelate dehydrogenase, but has no D-mandelate dehydrogenase. Mutants were selected by their ability to grow on D-mandelate, and in every case the D-mandelate dehydrogenase was also synthesized constitutively, although there had been no selective pressure on the parental strain which made this an advantage.

6.2.3 Measurement of the mandelate pathway enzymes in various mutants derived from wild-type strain NCIB8250

In the previous experiments, the assumption has been made that constitutivity in the various mutant strains is at the level of the R_1 regulon. To test the validity of this assumption all of the R_1 regulon enzymes had to be measured. A mutant from each class of constitutive mutants, the parent strains, and wild-type strain NCIB8250

were therefore tested. Other enzymes examined for possible altered regulation were 'benzoate oxidase' and catechol 1,2-oxygenase, two enzymes involved in mandelate metabolism below the level of benzoate. Benzyl alcohol dehydrogenase was measured as an indicator of R_2 regulon activity, and NADH oxidase as a measure of general enzymic activity.

The results in Table 18 show that constitutive strains growing on L-glutamate alone have activities of all R_1 regulon enzymes and NADH oxidase of similar order to the induced control strains. As expected, benzyl alcohol dehydrogenase activity is absent for the induced strains and this is also true of the constitutive mutants. Strains NCIB8250, 41 and D4OE possess 'benzoate oxidase' and catechol 1,2-oxygenase activity because they are utilizing the benzoate produced by phenylglyoxylate metabolism. However, the constitutive mutants do not possess these enzyme activities suggesting that constitutivity is confined to the R_1 regulon enzymes.

Although benzaldehyde dehydrogenase is one of the R_1 regulon enzymes, its activity was not routinely measured since, as Table 18 shows, the activity is very low. This confirms previous results (Livingstone & Fewson, 1972; Cook et al., 1975) which suggest that this is the rate-limiting enzyme responsible for the accumulation of benzaldehyde when bacteria are grown on L-mandelate or phenylglyoxylate alone.

6.2.4 Phenylglyoxylate carboxy-lyase activity in mutants isolated from strain 41 able to grown well on benzyl alcohol plates

During the isolation of constitutive mutants of strain 41 (Methods 8.1), 103 mutants were also cloned, which unlike the constitutive

Table 18 Activities of the enzymes of the mandelate pathway in various mutants derived from A. calcoaceticus NCIB8250

Wild-type strain NCIB8250 and mutants 41 and D40E were grown in 5mM-L-glutamate + 5mM-phenylglyoxylate/salts P2 medium at 23°C, and mutants HMM5,219 and D6 were grown in 5mM-L-glutamate/salts P2 medium at 23°C (Methods 6.8). The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted (Methods 9.1.1). The activities of D- and L-mandelate dehydrogenase, benzaldehyde dehydrogenase, NADH oxidase and catechol 1,2-oxygenase were assayed in triplicate using the extract prepared by ultrasonic disruption (Methods 11.1, 11.2.1, 11.4, 11.6 and 11.7). Phenylglyoxylate carboxy-lyase and benzyl alcohol dehydrogenase were assayed three times using toluenised bacterial suspensions (Methods 10, 11.3 and 11.5). Oxygen uptake, using benzoate as substrate, was measured in duplicate using suspensions of intact bacteria as described in Methods 15.1.

G + P : 5mM-L-glutamate + 5mM-phenylglyoxylate/P2 salts medium

G : 5mM-L-glutamate/salts P2 medium

Strain	Carbon source	Enzyme activity (munits (mg protein)$^{-1}$)							Oxygen uptake with benzoate (nmol O_2 min^{-1} (mg protein)$^{-1}$)
		DMDH	LMDH	PC	BDH	BADH	CO	NADH oxidase	
8250	G + P	0	93	614	14	0	197	52	71
41	G + P	79	90	597	27	0	151	51	103
D40E	G + P	62	0	642	12	0	232	64	101
HMM5	G	0	81	709	13	0	0	39	0
219	G	61	71	761	22	0	0	36	0
123	G	107	0	901	13	0	0	59	0
D6	G	97	126	1012	10	0	0	52	0

mutants, showed good growth on benzyl alcohol plates. Of these mutants, five (215, 224, 302, 308 and 331) were further characterized by growing on L-glutamate alone or L-glutamate in the presence of the following compounds: phenylglyoxylate; 2-phenylpropionate; phenylglyoxylate + 2-phenylpropionate. Parent strain 41, as well as constitutive mutant strains 218 and 219 were included as controls and the activity of phenylglyoxylate carboxy-lyase measured (Table 19). Mutants 215, 224, 302 and 308 behaved identically to the parent strain 41, possessing phenylglyoxylate carboxy-lyase activity only when grown on L-glutamate + phenylglyoxylate. Mutant 331, however, had phenylglyoxylate carboxy-lyase activity when grown on L-glutamate + phenylglyoxylate + 2-phenylpropionate, although the activity was low; this may suggest a mutation so that 2-phenylpropionate was a less effective anti-inducer.

The phenylglyoxylate carboxy-lyase activity after growth on L-glutamate + phenylglyoxylate + 2-phenylpropionate was examined in another eighteen mutants (Table 19). Low activities 16-92munits (mg protein)$^{-1}$ were found in ten of these mutants, therefore they resemble mutant 331 in having a lowered anti-induction by 2-phenylpropionate.

All the strains listed on Table 19 and an additional thirteen mutants (265, 271, 274, 278, 286, 289, 298, 305, 320, 330, 332, 336, and 345) failed to grow in 2mM-phenylpropionate medium showing that in none of these cases was there a mutation to allow growth on 2-phenylpropionate: this would be another possible way in which mutants might arise in the enrichment procedure. The nature of mutants like 331 remains unclear.

Table 19 Phenylglyoxylate carboxy-lyase activity in mutants isolated from strain 41 which are able to grow well on benzyl alcohol plates

Mutants were grown at 23°C in either 5mM-L-glutamate/salts medium (G) alone or the same medium containing the following additions; 2mM-phenylglyoxylate (G + P); 2mM-phenylpropionate (G + PP); 2mM-phenylglyoxylate + 2mM-phenylpropionate (G + P + PP) (Methods 6.4). The bacteria were harvested and stored as described in Methods 7.2. Phenylglyoxylate carboxy-lyase activity was determined in duplicate using toluenised bacterial suspensions (Methods 10 and 11.3).

Mutant	Phenylglyoxylate carboxy-lyase activity munits (mg protein)$^{-1}$			
	G	G+P	G+PP	G+P+PP
41	0	780	0	0
218	765	720	969	723
219	656	1018	1125	843
215	0	667	0	0
224	0	731	0	0
302	0	683	0	0
308	0	793	0	0
331	0	963	0	53
213				84
214				92
222				44
223				86
227				0
229				0
230				0
234				69
237				0
238				75
244				16
247				85
249				45
251				0
256				0
259				35
260				0
262				0

The results in Table 19 confirm the effectiveness of the screening technique using growth on benzyl alcohol plates; since none of the strains tested showed synthesis of phenylglyoxylate carboxy-lyase in the presence of phenylglyoxylate + 2-phenylpropionate, this would suggest that all mutants constitutive for phenylglyoxylate carboxy-lyase were recognised in the isolation procedure.

7. Isolation of mutants from A. calcoaceticus EBF65/65, mutant strain C48

Strain C48 is a double auxotroph derived from wild-type strain EBF65/65. This mutant can metabolize D-mandelate dehydrogenase (see later : Table 21), but lacks L-mandelate dehydrogenase activity.

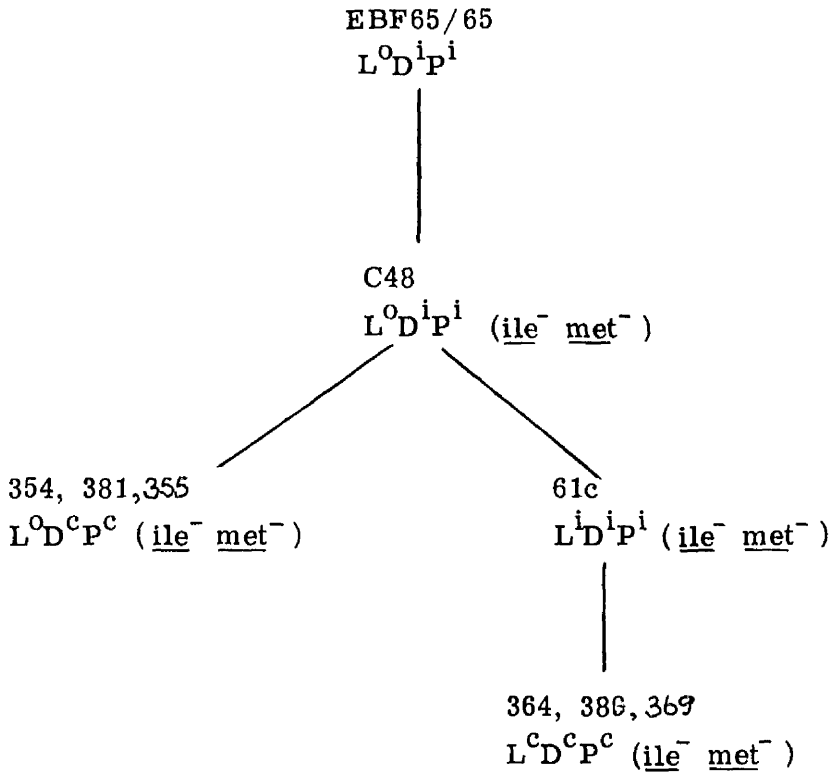
Mutants were isolated from this strain with the ability to utilize L-mandelate (see Scheme 13). Table 20 shows the frequency of appearance of mutants on solid and liquid medium containing L-mandelate. The frequency of mutation was estimated using the expression:

$$\text{Frequency of mutation} = \frac{\text{number of mutants}}{\text{number of bacteria capable of mutation}}$$

The frequency of spontaneous and NTG-induced mutation on solid medium was the same. However, the frequency of mutation in liquid medium appeared to be an order of magniture lower.

One of these mutants, 61c (see Fig. 19) , isolated from solid medium as well as strain C48 were then used as parent strains for isolation of mutants constitutive for phenylglyoxylate carboxy-lyase synthesis (Methods 8.1). Three constitutive mutants were isolated from each strain (Table 3 in Methods; see Scheme 13).

Fig. 19 shows the growth of mutants 354 and 364, and the parent strains C48 and 61c respectively, on agar plates containing L-mandelate, D-mandelate or phenylglyoxylate + 2-phenylpropionate. All four strains are still auxotrophic for isoleucine and methionine and capable of utilizing D-mandelate, whereas only strains 61c and 364 grow on L-mandelate. Furthermore mutants 354 (derived from C48)



L L-mandelate dehydrogenase

D D-mandelate dehydrogenase

P phenylglyoxylate carboxy-lyase

o no enzyme activity

i inducible enzyme activity

c constitutively synthesized enzyme activity

(ile⁻ met⁻) isoleucine + methionine requiring auxotrophs

MUTANTS ISOLATED FROM Acinetobacter calcoaceticus EBF65/65

Scheme 13

Table 20 Frequency of appearance of mutants of strain C48 with the ability to grow on L-mandelate

Various volumes (as described in Methods 8.3) of a nutrient broth culture grown for 24h at 30°C were inoculated into nineteen flasks containing 5mM-L-mandelate/salts medium supplemented with D,L-isoleucine + L-methionine ($0.01 g l^{-1}$ and $0.005 g l^{-1}$ respectively). The cultures (50ml in 250ml conical flasks) were grown at 30°C on a rotary shaker, and the number of flasks showing growth monitored for 5d. Ten plates containing the same medium were spread with the same culture, a few crystals (1-5) of NTG placed in the centre of five of them.

It was assumed that there were 10^8 bacteria ml^{-1} nutrient broth culture

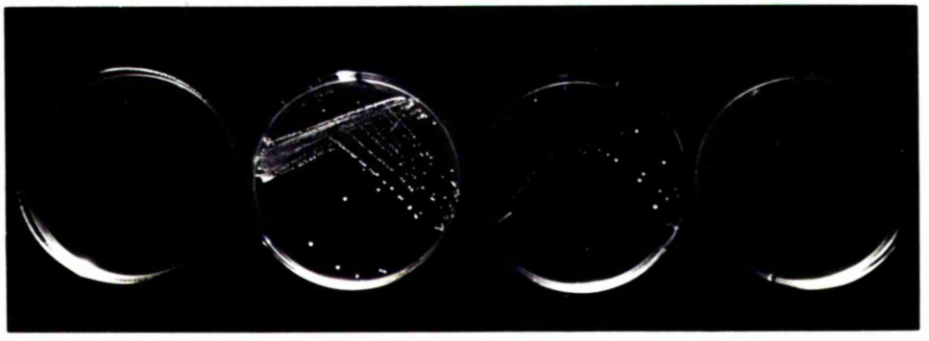
Medium	Mutagen	Number of bacteria capable of mutation	Number of mutants	Estimated frequency of occurrence of mutants
Solid	None	5×10^{7}	2	4×10^{-8}
Solid	NTG	5×10^{7}	2	4×10^{-8}
Liquid	None	3.7×10^{9}	14*	3.8×10^{-9}

* i.e. 14 out of 19 flasks showed growth

Fig. 19 Growth of mutants of strain EBF65/65 on solid medium containing L-mandelate, D-mandelate, or phenylglyoxylate + 2-phenylpropiona

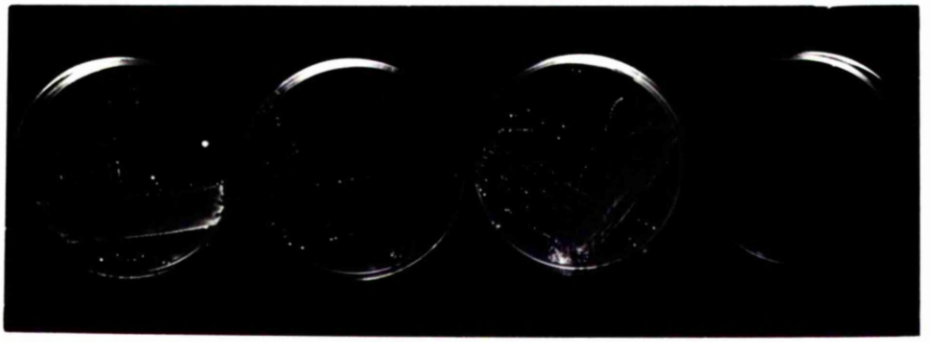
Nutrient broth cultures of mutants C48, 354, 61c and 364 we grown for 24h at 30°C. The bacteria were spread on plates (9cm diameter) of salts media containing agar and 2mM-L-mandelate, 2mM-D-mandelate, or 2mM-phenylglyoxylate + 2mM-2-phenylpropionat These plates were supplemented with D,L-isoleucine + L-methionin ($0.05 g l^{-1}$ of each). In addition mutants C48, 354 and 364 were spread on similar plates containing 2mM-D-mandelate, and mutant 61c on 2mM-L-mandelate, but no isoleucine or methionine was added. The plates were incubated at 30°C for 48h and then stored at room temperature for several days before being photographed. During this time they were opened and an obvious contaminant appeared on the plate containing L-mandelate + isoleucine + methionine spread with mutant C48 (see plate top left hand corner).

C48



354

61c



364

L-mandelate + ile met

D-mandelate + ile met

Phenyglyoxylate + Phenylpropionate + ile met

No ile met

and 364 (derived from 61c) grow in the presence of 2-phenylpropionate whilst the parental strains do not.

7.1 Measurement of the enzymes of the mandelate pathway in mutantsderived from strains C48 and 61c

Table 21 shows the enzyme activities for mandelate metabolism to the level of cis,cis-muconate and NADH oxidase measured in wild-type strain EBF65/65 and mutant strains derived from strains C48 and 61c.

Comparison of enzyme activities measured in strain C48 in the presence and absence of phenylglyoxylate emphasizes the inducible nature of all the enzymes except NADH oxidase. In contrast, mutants isolated from this strain on the basis of their ability to synthesize phenylglyoxylate carboxy-lyase constitutively also synthesize D-mandelate dehydrogenase and benzaldehyde dehydrogenase constitutively when grown on the non-inducing carbon source, L-glutamate. Similarly mutants isolated from strain 61c synthesize these three enzymes as well as L-mandelate dehydrogenase constitutively. This latter enzyme is inducible in parent strain 61c, as are the other enzymes of the pathway.

In these six mutants it can be seen that only those enzymes involved in the metabolism of D- or L-mandelate to the level of benzoate are synthesized constitutively.

Table 21 Activities of the enzymes of the mandelate pathway in mutants derived from strains 61c and C48 which synthesize phenylglyoxylate carboxy-lyase constitutively

Wild-type strain EBF65/65, mutants 61c and C48 were grown in 5mM-L-glutamate + 5mM-phenylglyoxylate/salts P2 medium supplemented with D,L-isoleucine + L-methionine whilst mutants 354, 355, 364, 369, 381 and 386 were grown in 5mM-L-glutamate/salts P2 medium supplemented with D,L-isoleucine + L-methionine. The bacteria were grown at 23°C as described in Methods 6.11, harvested, washed and stored (Methods 7.1 and 7.2). The activities of the following enzymes were measured in triplicate using extracts prepared by ultrasonic disruption (Methods 9.1.1); D- and L-mandelate dehydrogenase; benzaldehyde dehydrogenase; NADH oxidase; catechol 1,2-oxygenase (Methods 11.1, 11.2.1, 11.4, 11.6 and 11.7). Phenylglyoxylate carboxy-lyase activity was measured three times in toluenised bacterial suspensions (Methods 10 and 11.3), and oxygen uptake, using benzoate as substrate, was assayed in duplicate using suspensions of intact bacteria as described in Methods 15.1.

G + P : 5mM-L-glutamate + 5mM-phenylglyoxylate/salts P2 medium + D,L-isoleucine + L-methionine

G : 5mM-L-glutamate/salts P2 medium + D,L-isoleucine + L-methionine

Mutant	Carbon source	Enzyme activity (munits (mg protein)$^{-1}$)						Oxygen uptake with benzoate (nmol O_2 min^{-1} (mg protein)$^{-1}$)
		DMDH	LMDH	PC	BDH	CO	NADH oxidase	
EBF65/65	G + P	81	0	440	10	203	50	437
C48	G	0	0	0	0	0	37	0
C48	G + P	66	0	383	9	97	71	131
354	G	111	0	552	36	0	63	0
355	G	44	0	292	11	0	88	0
381	G	12	0	79	3	0	59	0
61c	G + P	76	39	489	17	158	93	161
364	G	138	56	698	29	0	85	0
369	G	52	23	315	9	0	80	0
386	G	28	12	132	4	0	73	0

8. Comparison of the D- and L-mandelate dehydrogenases from mutants 219 and 364

Although mutants 219 (ultimately derived from wild-type NCIB8250) and 364 (ultimately derived from wild-type EBF65/65) possess both L- and D-mandelate dehydrogenase activities, in the former the D-mandelate dehydrogenase is a newly evolved enzyme, whilst in the latter L-mandelate dehydrogenase has been evolved. A preliminary comparison of the original and newly evolved enzymes was thus possible using these two mutants derived originally from the two different strains of A. calcoaceticus.

Table 22 shows that the spectrophotometric measurement of activities of both original and evolved D-mandelate dehydrogenases was absolutely dependent upon the inclusion of PMS and BSA in the reaction mixture. Although the activities of both original and evolved L-mandelate dehydrogenases were not strongly dependent upon the presence of PMS and BSA, addition of these components to the reaction mixture gave similar enhancement of activity for both enzymes. Inclusion of BSA alone, however, had an inhibitory effect on the activity of both L-mandelate dehydrogenases.

A further comparison of the enzymes was made by measuring the loss of enzyme activity following incubation of extracts of strain 219 and 364 at 23°C and 30°C (Fig. 20). Both original and evolved L-mandelate dehydrogenase activities remained relatively stable at both temperatures. In contrast, both D-mandelate dehydrogenases were heat-labile, showing greater lability at 30°C. At each temperature the lability of both original and evolved D-mandelate dehydrogenases were similar.

Table 22 The effect of omitting reagents from the reaction mixtures used to measure D- and L-mandelate dehydrogenase activity for mutants 219 and 364

Mutant 219 was grown at 23°C in 5mM-L-glutamate/salts P2 medium (Methods 6.8) and mutant 364 was grown in similar medium supplemented with D,L-isoleucine + L-methionine (Methods 6.11). The bacteria were harvested, washed, stored, and ultrasonically disrupted using soniprobe number 1 as described in Methods 7.1 and 9.1.1. The activities of D- and L-mandelate dehydrogenases were assayed when various components of the reaction mixture were omitted.

n.d. = not determined

Strain 219 (derived from strain 41)

Reaction component omitted	Enzyme activity munits (mg protein)$^{-1}$	
	DMDH	LMDH
None	48	75
BSA	5	68
PMS	12	18
BSA, PMS	n.d.	56
substrate	10	9
extract	9	6

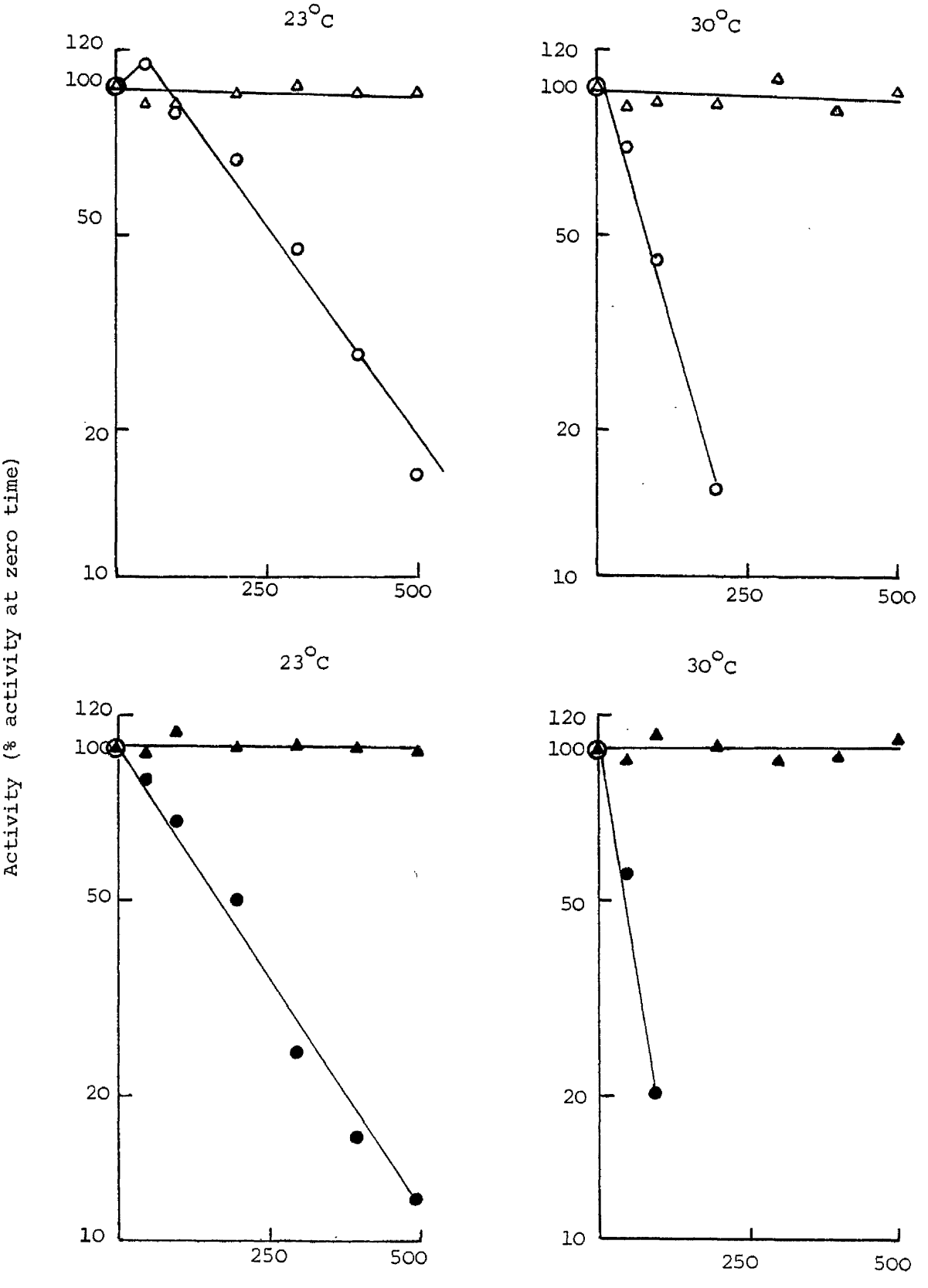
Strain 364 (derived from strain 61c)

Reaction component omitted	Enzyme activity munits (mg protein)$^{-1}$	
	DMDH	LMDH
None	198	107
BSA	14	83
PMS	0	21
BSA, PMS	n.d.	64
substrate	13	15
extract	13	10

Fig. 20 Heat inactivation of D- and L-mandelate dehydrogenase of mutants 219 and 364

Mutant 219 was grown at 23°C in 5mM-L-glutamate/salts P2 medium (Methods 6.8) and mutant 364 was grown in similar medium supplemented with D,L-isoleucine + L-methionine (Methods 6.11). The bacteria were harvested, washed, stored (Methods 7.1) and ultrasonically disrupted using probe number 1 (Methods 9.1.1). The extracts were incubated at 23°C and 30°C and samples were withdrawn at intervals for the determination of D- and L-mandelate dehydrogenase activity (Methods 11.1 and 11.2.2). The activity of each enzyme is expressed as a percentage of the activity at zero time. Values below 10% of the starting activity have not been included.

	Mandelate dehydrogenase	Mutant strain	Activity at zero time munits (mg protein)$^{-1}$
o	DMDH	219	57
Δ	LMDH	219	90
●	DMDH	364	164
▲	LMDH	364	51


23°C
30°C
23°C
30°C
120
100
50
20
10
250
500
Activity (% activity at zero time)
Time of incubation (min)

9. Enrichment of mutants with evolved mandelate dehydrogenases from strains NCIB8250, NF1408 and C48

Previous results (Section 8) suggest similarities between the evolved D-mandelate dehydrogenase in mutant strains derived from NCIB8250 and the original D-mandelate dehydrogenase in strain EBF65/65. This raises the possibility that both strain NCIB8250 and EBF65/65 possess a similar D-mandelate dehydrogenase gene which, however, is not expressed in NCIB8250. If there is a 'silent' gene, then the rate of mutation to D-mandelate utilization in strain NCIB8250 should be similar to that found for strain NF1408, a mutant lacking L-mandelate dehydrogenase activity.

Since the evolved L-mandelate dehydrogenase of strain EBF65/65 is similar to the original enzyme in strain NCIB8250 a similar suggestion of a 'silent' gene coding for L-mandelate dehydrogenase in strain EBF65/65 can be made.

9.1 Estimation of frequency of mutation of strains NCIB8250 and NF1408 to utilize D-mandelate, and strain NF1408 to utilize L-mandelate

The frequency of occurrence of mutants was estimated using the following expression:

$$\text{Frequency of occurrence of mutants} = \frac{\text{Number of flasks showing growth}}{\text{Total number of bacteria capable of mutation}}$$

The assumption made in this calculation was that growth in each flask was due to one mutant only. This assumption was probably correct when only a small proportion of the flasks showed growth.

When enriching for mutation to D-mandelate utilization in strain NF1408, the cultures were incubated at 23°C and 30°C. Since D-mandelate dehydrogenase is heat-labile there was a possibility that more mutants would appear at 23°C, however Table 23 shows that the observed frequency of occurrence of mutants was about the same at both temperatures. In strain NF1408 the frequency of appearance of mutants able to utilize D-mandelate was about five times greater than the appearance of mutants with the ability to utilize L-mandelate. Also the frequency of appearance of mutants able to utilize D-mandelate in strain NF1408 was about ten times greater than in strain NCIB8250. More experiments would have to be carried out in order to determine if these differences were significant.

9.2 Summary of frequency of appearance of mutants from strains NCIB8250, NF1408 and C48 able to utilize D- or L-mandelate

My results for strains NCIB8250 and NF1408 are compared with those of other workers in Table 24. For strain NCIB8250, the frequencies of occurrence of mutants utilizing D-mandelate are, with one exception, in close agreement when D-mandelate supplemented with phenylglyoxylate was used for enrichment. The estimated frequency in the absence of phenylglyoxylate was an order of magnitude higher, but the assumption of 10^8 bacteria per ml of nutrient broth may be an underestimate.

For strain NF1408 the frequencies of occurrence of mutants able to utilize D-mandelate were in good agreement and were similar to those found for strain NCIB8250.

A lower value was obtained for reversion of NF1408 to the ability to utilize L-mandelate when the enrichment medium was supplemented with phenylglyoxylate. However, in the absence of phenylglyoxylate the

Table 23 Estimation of frequency of mutation of strains NCIB8250 and NF1408 to gain the ability to utilize D-mandelate and of strain NF1408 to gain the ability to utilize L-mandelate

Various volumes (as described in Methods 8.2, Table 5) of nutrient broth cultures of strains NCIB8250 and NF1408 grown for 24h at 30°C and a culture of NF1408 grown for 48h at 23°C were used to inoculate 5mM-D-mandelate + 0.25mM-phenylglyoxylate/salts medium. Similarly, various volumes of a culture of NF1408 grown for 24h at 30°C were used to inoculate 5mM-L-mandelate + 0.25mM-phenylglyoxylate/salts medium. The cultures (50ml in 250ml conical flasks) were grown at 23°C or 30°C on a rotary shaker and the number of flasks showing growth monitored for 5d.

The number of bacteria in the enrichment media capable of mutation were estimated by growing the strains in 0.25mM-phenylglyoxylate/salts medium for 24h, i.e. with no extra carbon source. These cultures were then serially diluted and spread on nutrient agar plates and the number of colonies counted.

The estimated frequency of occurrence of mutants was calculated as described in the text.

D + P 5mM-D-mandelate + 0.25mM-phenylglyoxylate/salts medium

L + P 5mM-L-mandelate + 0.25mM-phenylglyoxylate/salts medium

Starting strain	Enrichment medium	Temp. of growth (^{o}C)	Total number of bacteria capable of mutation	Number of flasks inoculated	Number of flasks showing growth	Estimated frequency of occurrence of mutants
NCIB8250	D + P	30	2.2×10^{11}	39	1	4.5×10^{-12}
NF1408	D + P	30	2.3×10^{11}	39	11	4.8×10^{-11}
NF1408	D + P	23	4.3×10^{11}	39	14	3.2×10^{-11}
NF1408	L + P	30	2.3×10^{11}	39	2	8.7×10^{-12}

frequency of mutation appears an order of magnitude greater. Again this suggested that the value assigned to the total number of bacteria capable of mutation was too low.

Included in Table 24 are results already presented in Table 20 for the frequency of occurrence of mutants of C48 able to utilize L-mandelate. The frequency of appearance of these mutants was at least one or two orders of magnitude higher than the frequency of appearance of mutants of NCIB8250 or NF1408 able to utilize D-mandelate (when also enriched in the absence of phenylglyoxylate).

Table 24 Summary of the estimation of frequency of mutation of strains NCIB8250 and NF1408 to gain the ability to utilize D-mandelate and of strains NCIB8250 and C48 to gain the ability to utilize L-mandelate

Various volumes (as described in Methods 8.2, 8.3 and Table 5) of nutrient broth cultures of strains NCIB8250, NF1408 and C48 grown for 24h at 30°C and a culture of NF1408 grown for 48h at 23°C were used to inoculate the following (as appropriate):

5mM-D-mandelate/salts medium (D)

5mM-D-mandelate + 0.25mM-phenylglyoxylate/salts medium (D + P)

5mM-L-mandelate/salts medium (L)

5mM-L-mandelate + 0.25mM-phenylglyoxylate/salts medium (L + P)

5mM-L-mandelate/salts medium supplemented with D,L-isoleucine + L-methionine ($0.01 gl^{-1}$ and $0.005 gl^{-1}$ respectively; L + ile + met)

The cultures (50ml in 250ml conical flasks) were incubated at 23°C or 30°C on a rotary shaker and the number of flasks showing growth monitored for 5d.

The number of bacteria in the enrichment media containing phenylglyoxylate was based upon the number I had estimated previously (see legend to Table 23). When phenylglyoxylate was omitted from the enrichment medium it was assumed that there were 10^8 bacteria ml^{-1} of nutrient broth culture.

J.D.B. : J.D. Beggs (Lancaster, 1971)

C.A.F. : C.A. Fewson (unpublished work)

C.A.H. : C.A. Hills (this thesis)

* : results from Table 23 of this thesis

† : results from Table 20 of this thesis

Where more than one estimation of the frequency of occurrence of mutants exists, the number of flasks showing growth and the number of bacteria capable of mutation in each estimation have been summed and the total values used to calculate an overall frequency of occurrence of mutants. This figure is shown in parentheses.

Starting strain	Enrichment medium	Temp. of growth (°C)	Total number of bacteria capable of mutation	Number of flasks inoculated	Number of flasks showing growth	Estimated frequency of occurrence of mutants	Experiment of
NCIB8250	D + P	30	1.8×10^{11}	32	7	3.9×10^{-11}	C.A.F.
		30	4.5×10^{10}	8	1	2.2×10^{-11}	J.D.B.
		30	2.2×10^{11}	40	5	2.2×10^{-11}	C.A.H.
		30	2.2×10^{11}	39	1	4.5×10^{-12}	C.A.H.*
						(2.1×10^{-11} total)	
NCIB8250	D	30	7.5×10^{9}	40	5	6.7×10^{-10}	C.A.H.
NF1408	D + P	30	1.9×10^{11}	32	13	6.8×10^{-11}	C.A.F.
		30	2.3×10^{11}	39	11	4.8×10^{-11}	C.A.H.*
						(5.7×10^{-11} total)	
NF1408	D + P	23	4.3×10^{11}	39	14	3.3×10^{-11}	C.A.H.*
NF1408	L + P	30	1.9×10^{11}	32	0	$< 5.3 \times 10^{-12}$	C.A.F.
		30	2.3×10^{11}	39	2	8.7×10^{-12}	C.A.H.*
		30	2.3×10^{11}	39	4	1.7×10^{-11}	C.A.H.
						(9.2×10^{-12} total)	
NF1408	L	30	7.4×10^{9}	38	1	1.4×10^{-10}	C.A.H.
C48	L + ile + met	30	3.7×10^{9}	19	14	3.8×10^{-9}	C.A.H.†

D I S C U S S I O N

1. Lysis of cultures

Growth of strain 41 and mutants derived from it (e.g. mutant 219) was complicated by the occasional, but apparently random, occurrence of lysis. Initially it was thought that lysis could be due to some disturbance of the bacterial membrane by the newly acquired D-mandelate dehydrogenase, which appears to be membrane-bound. This suggestion seems unlikely, however, since other strains possessing D-mandelate dehydrogenase activity (either evolved, as in mutants such as D4OE and 123 derived from strain NCIB8250, or original as in strain EBF65/65) did not lyze. Conversely, strain 41 sometimes lyzed under conditions where D-mandelate dehydrogenase was not induced and so presumably the enzyme protein was not present. It is of course possible, but improbable, that it was only by chance that lysis was not seen in these apparently less susceptible strains simply because they were not grown as often as strains 41 and 219. However, lysis of wild-type strain NCIB8250 and mutant HMM5, which do not have D-mandelate dehydrogenase activity, has recently been observed by Dr C.A. Fewson under conditions of fast stirring (unpublished results), so this phenomenon does not appear to be peculiar to mutants 41 and 219, although these strains may be more susceptible to it. It is possible that as strain 41 was isolated from wild-type strain NCIB8250 in the presence of NTG, there might have been one or more additional mutations in genes involved in some aspect of the structural integrity of the bacterium as well as the mutation giving D-mandelate dehydrogenase activity. Guerola *et al.* (1971), for instance have shown that NTG can induce multiple mutations which are closely linked. In any case it is clear that the presence of D-mandelate dehydrogenase is not in

itself sufficient to cause lysis because static nutrient broth cultures of mutant 219 never lyze (p.87). In addition, as mentioned above, lysis sometimes occurred in mutant 41 when it was uninduced for D-mandelate dehydrogenase. In other words, factors such as composition of the medium and rate of stirring may contribute to the effect.

Other strains of Acinetobacter have been seen to lyze due to the presence of lytic phages (Herman & Juni, 1974). In the present case, no lysis of bacterial lawns of either strains NCIB8250 or 41 was observed with samples of lyzed culture (Results 1.1) suggesting that phages were not responsible for lysis in this case. However, if the wild-type strain NCIB8250 was lysogenic, then no lysis of this strain, or any strains derived from it, would necessarily be observed with added phage, and so this experiment was not entirely conclusive.

Although the cause of lysis is not fully understood, its occurrence was largely overcome (Results 1.1) and reasonable yields obtained, by using a large nutrient broth inoculum and growing the bacteria for relatively short times in defined media. This proved satisfactory for the work presented in this thesis although the experiments involving the induction, anti-induction and repression of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase (Table 15 Fig. 17) were less elegant than similar experiments of Beggs & Fewson (1977). These workers used nutrient broth cultures to inoculate L-glutamate/salts medium which in turn was used to inoculate L-glutamate/salts medium containing the appropriate inducers or repressors. In this way the bacteria were adapted to growth on the non-inducing carbon source, L-glutamate, before the appropriate inducers or repressors were present.

2. The initial step in the metabolism of D-mandelate

2.1 The existence of a specific D-mandelate dehydrogenase

The following possibilities to account for the metabolism of D-mandelate in mutants of wild-type NCIB8250 were considered:

(a) D-mandelate —DMDH / no LMDH→ phenylglyoxylate

(b) D-mandelate / or / L-mandelate —non-specific dehydrogenase→ phenylglyoxylate

(c) D-mandelate —DMDH→ phenylglyoxylate; L-mandelate —LMDH→ phenylglyoxylate

(d) D-mandelate —racemase→ L-mandelate —LMDH→ phenylglyoxylate

(e) D-mandelate —?→ X

In the simplest terms: (a) involves alteration of L-mandelate dehydrogenase to D-mandelate dehydrogenase, i.e. a change to the opposite stereospecificity; (b) only requires loss of stereospecificity of L-mandelate dehydrogenase to give a non-specific dehydrogenase; both (c) and (d) require a new enzyme, a D-mandelate dehydrogenase or a racemase respectively; (e) would involve a new pathway, possibly with several new enzymes, for example involving similar intermediates and enzymes to those found in the mandelate pathway of *P. convexa* (Scheme 6).

The isolation of the first strains which could grow on D-mandelate, e.g. 41 (Lancaster, 1971), eliminated possibility (a) because all evolvants could oxidize both L- and D-mandelate, and so there could not have been a total change from L-mandelate dehydrogenase to D-mandelate dehydrogenase activity. This was confirmed when strain 41 was shown to have both L- and D-mandelate dehydrogenase activity (Table 15).

Ahlquist (1974) constructed, by transformation, strains D4OE and D4OG which could grow on only D-mandelate, and not the L-isomer, and so possibilities (b) and (d) were ruled out. This was confirmed when strain D4OE, as well as constitutive strains derived from this strain (see e.g. Table 17b) were shown to have D-mandelate dehydrogenase, but not L-mandelate dehydrogenase activity. Further, no racemization of L-mandelate was detected in mutant 41 (Matson, 1974), which again eliminates possibility (d).

Of the remaining possibilities, (c) seemed more likely. Very strong evidence for this was provided when phenylglyoxylate was shown to be the only identifiable product of D-mandelate oxidation (Results 2.1). The subsequent finding that stoicheiometrically equivalent amounts of phenylglyoxylate were obtained by D-mandelate oxidation (Fig. 6) implied that this was the only pathway operating. Since the initial product of D-mandelate utilization appears to be phenylglyoxylate, it seems likely that the enzyme involved must be a dehydrogenase. Indeed with a membrane preparation and D-mandelate as substrate the stoicheiometry of oxygen uptake is consistent with dehydrogenation to phenylglyoxylate (Results 4). Furthermore, the reduction of the hydrogen acceptors

DCIP and PMS in the oxygen electrode (Fig. 11) and the spectrophotometric assay (Results 3) imply the presence of a dehydrogenase.

Additional evidence to support the pathway (c) is that, during growth on D-mandelate, phenylglyoxylate must be formed to induce the rest of the R_1 regulon enzymes (e.g. Table 15, 4) and secondly benzaldehyde can be detected. There is no evidence to suggest another enzyme and so possibility (e) can be eliminated.

Similar lines of reasoning suggest that mutants of strain EBF65/65 can grow on L-mandelate by virtue of an evolved L-mandelate dehydrogenase.

2.2 The D-mandelate dehydrogenase assay

Application of the original technique for measuring L-mandelate dehydrogenase to the measurement of D-mandelate dehydrogenase activity proved unsuccessful unless both BSA and PMS were included in the reaction mixture (Fig. 7 & 8, Table 8). A comparison of the results obtained for oxygen uptake with those found using the spectrophotometric assay allows some speculation concerning the role of these two assay components. This comparison is justifiable since the rate of DCIP reduction observed spectrophotometrically was shown to be equivalent to the rate of oxygen uptake in studies using the same membrane preparation (see p.121).

PMS presumably acts as an intermediate electron carrier since the reaction involved is a dehydrogenation. Evidence consistent with this role is: (a) the enhancement of oxygen uptake by PMS in the presence or absence of reduced DCIP (Table 12 v & iv); (b) inclusion of PMS, even without BSA, in the D-mandelate dehydrogenase assay gives an enhanced rate of DCIP reduction provided that large amounts of extract are used (Fig. 8), suggesting that reduced PMS becomes reoxidized by a process which eventually reduces DCIP; (c) the increase in rate of DCIP

reduction seen on addition of PMS to the L-mandelate dehydrogenase assay (Fig. 12).

Reduced DCIP inhibits oxygen uptake in the presence or absence of PMS (v & iii, Table 12). It seems likely that reduced DCIP is also responsible for the decrease in the rate of L- and D-mandelate dehydrogenase activity with respect to time when measured spectrophotometrically (see p.107). Originally it was thought that this fall off in reaction rate was due to oxidation of reduced DCIP by either (a) auto-oxidation in the presence of oxygen, or (b) enzymic oxidation e.g. by cytochrome oxidase. However lack of enhancement of mandelate dehydrogenase activity under anaerobic conditions (p.109) eliminates this possibility and strengthens the idea that reduced DCIP is inhibitory.

Thus, reduced DCIP appears to inhibit the reduction of DCIP itself. This inhibition appears to be abolished by the addition of BSA: (a) approximately the same rate of oxygen uptake was seen with BSA and PMS in the presence of reduced DCIP as with PMS alone (Table 12 vi compared with iv); (b) the rate of DCIP reduction during the measurement of L- or D-mandelate dehydrogenase activity was linear for at least 4min in the presence of BSA (p.109 & 124). Alleviation of inhibition by reduced DCIP would appear to be one role of BSA. However, the picture is complicated by the observations that when PMS is absent BSA (a) prevents the preferential reduction of DCIP in the oxygen electrode (p.121) and (b) prevents the reduction of DCIP in the spectrophotometric assay (see Table 22).

Thus although BSA seems very important in the D-mandelate dehydrogenase assay its mode of action is not absolutely clear. BSA is certainly used in many enzyme assays but the reason for its inclusion

is not generally stated and probably not known. In fact, the spectrophotometric assay for mitochondrial succinate dehydrogenase, a membrane-bound enzyme, is remarkably similar to the assay for D-mandelate dehydrogenase in that the reduction of DCIP is followed in the presence of 0.36mg PMS and 7mg BSA per ml (Hatefi, 1978).

Several possibilities may be considered for the action of BSA. Firstly, BSA is a protein and when present in the relatively high amounts used [although the concentration is only 50μM, the monomeric molecular weight being about 68,000 (see Peters & Reed, 1978)], it may give some form of protection to the enzyme. This appears unrelated to any protection against proteolytic activity which may be degrading D-mandelate dehydrogenase (p.104). Some sort of protein-protein interaction therefore seems likely. The enhancement of reaction rate when large quantities of extract were used without BSA (Fig. 8) may reflect such a protein-dependent protection. This possibility could be further explored by using proteins other than BSA, e.g. lysozyme.

Secondly, since most commercial BSA preparations contain variable quantities of lipid (e.g. see Chen, 1967), it is also possible that the enhancement of activity was due to the presence of lipids, especially as D-mandelate dehydrogenase appears to be membrane-bound. This does not seem to be the case, however, as removal of lipid by charcoal treatment (Methods 16) has no effect on enzyme activity (p.104). A commercial preparation of defatted BSA, and a crystalline sample of BSA were also tested (results not shown), but gave the same results as the powdered BSA normally used.

Thirdly, it is possible that stimulation of enzyme activity may be attributable to the free sulphydryl group of BSA (see Foster, 1960).

BSA has been found to stimulate the activity of chick embryo collagen proline hydroxylase (Propenoe et al., 1969; Rhoads et al., 1967). There is no entirely satisfactory explanation for this stimulation although evidence from chemical modification of BSA suggests that part, but not all, may be attributable to the free sulphydryl group of BSA (Propenoe et al., 1969). The presence of free sulphydryl groups could have an effect on the D-mandelate dehydrogenase since inhibition studies with p-chloromercuribenzoate and $HgCl_2$ (Table 14) suggest that this enzyme requires a sulphydryl group for activity. This possibility could be further investigated by seeing if other thiol-containing proteins such as glyceraldehyde-3-phosphate dehydrogenase can replace BSA. Unfortunately it is not possible to replace BSA by compounds such as dithiothreitol, mercaptoethanol or reduced glutathione because they chemically reduce DCIP. L-Mandelate dehydrogenase also requires a thiol group for activity (Table 14), but BSA is not absolutely required in the assay of this enzyme. However, the observations that L-mandelate dehydrogenase is less sensitive than D-mandelate dehydrogenase to p-chloromercuribenzoate (Table 14), as well as being less helped by BSA, may be consistent with each other.

Fourthly, BSA is known to have a number of binding sites which fall into the categories of: (a) hydrophobic, non-covalent sites for (i) primary long chain fatty acids, (ii) bilirubin and certain drugs, (iii) indole compounds and several other drugs; (b) covalent attachment sites for organic ligands; and (c) chelation sites for divalent metals (Peters & Reed, 1978). Any of these unique binding properties might account for its need in an enzyme assay (Taylor, 1977). BSA might be able to remove potentially inhibitory reaction products such as reduced DCIP. Indeed, if oxidised DCIP were also bound then this could explain

the apparent inhibition of DCIP reduction in the absence of PMS. To be consistent, however, the bound DCIP would need to be freely accessible for reduction by PMS. Alternatively BSA may protect the enzyme from inhibition by hydrophobic species in the reaction medium, although as the enzyme is membrane-bound hydrophobic molecules are probably less likely to be inhibitory.

In summary, BSA seems to exert two important effects (a) prevention of inhibition by reduced DCIP and (b) enhancement of electron transfer from PMS to DCIP, but not from the indigenous electron acceptor. As a consequence of (b), PMS has the valuable property of acting as an intermediate electron carrier, receiving electrons either from the indigenous electron acceptor (whatever that is) or by participating directly in the dehydrogenation reaction.

2.3 Reliability of the D-mandelate dehydrogenase assay

With an assay system available, it became possible to examine D-mandelate dehydrogenase activity in wild-type and mutant strains. For practical reasons, however, it was not possible to grow all the strains and assay for enzyme activity on the same day. Harvested bacteria were routinely stored at $-18^{\circ}C$ prior to preparation of extract and assay of enzyme activity. To justify the use of these results for comparative purposes it was necessary to establish the possible errors introduced by (a) growing bacteria on different occasions, (b) freezing and storing bacteria, (c) preparation of extracts and assaying enzyme activity on different occasions.

The assay procedure was shown to have small inherent variability when disruption and assay were repeated on the same day (Table 10a & b),

the standard deviation being about 7% of the mean. Table 9a & b indicates that growth of bacteria on different days, and freezing and storing for up to a week gives a range of measured activities with a standard deviation of about 7 to 10% of the mean (preparation of extract and assays were done on the same day), so relatively little additional error was introduced compared with that inherent in the disruption and assay procedure itself. Table 11 reflects the errors due to both freezing and storing the bacteria and carrying out the assay procedure on different occasions. These results have a standard deviation of about 10% of the mean, again showing little extra variability compared to that of the disruption and assay procedure. Thus reasonably small additional variability appeared to be introduced by the three possible sources of error considered.

An important consideration when comparing results of different experiments was the actual soniprobe used for disruption. Each soniprobe gave reproducible results, but the results differed for the two machines (e.g. compare the relatively low activities obtained in Table 10a & b after disruption of mutant 219 with probe no. 1, with those in Table 9 & 11 obtained after disruption of similar bacteria with probe no. 3). This difference between the machines is not surprising in view of the importance of precise conditions of current and time (Table 7). In all cases where comparisons have been made, however, the same soniprobe was used.

It may be remarked here that, theoretically, no direct correlation between measured enzyme activity and enzyme synthesis should be made as little is known concerning possible enzyme degradation or inactivation or, indeed, if it is synthesized as an active enzyme or as some inactive

precursor. Also, the enzyme activity may not be linearly related to the actual amount of enzyme protein. The assumption that enzyme activity is directly proportional to enzyme protein is often made too glibly and the results of this thesis showing the lability of D-mandelate dehydrogenase at 30°C demonstrate how false this assumption may be.

To summarize, the results obtained in Tables 17a, b & c, 18 & 21 may be confidently used to compare enzyme activity in the different mutants examined, and it is hoped that this reflects the total enzyme protein but this remains to be proved.

3. Comparison of the mandelate dehydrogenases

L-Mandelate dehydrogenase and D-mandelate dehydrogenase are very similar in many respects e.g.:

(a) Location in the membrane, not the soluble fraction.

(b) Require a thiol group for activity.

(c) Have little, if any, requirement for metal ions.

(d) Show a similar degree of inhibition by oxalate.

(e) Have similar pH and temperature-dependence for activity.

(f) Are completely stereospecific for the substrate.

(g) Are inhibited by the opposite isomer of mandelate

(h) Use the same range of electron acceptors (e.g. DCIP and PMS).

Kennedy & Fewson (1968b) suggested that L-mandelate dehydrogenase was a membrane-bound enzyme whilst phenylglyoxylate carboxy-lyase was soluble. This distinction was made on the basis of centrifugation experiments and pattern of release of activity during ultrasonic disruption. Likewise in the present work D- and L-mandelate dehydrogenase were found to behave identically, and in a manner consistent with their both being membrane-bound enzymes, during various ultrasonic treatments (Table 7) and centrifugation at 100,000g for 2.5h (results not shown); conversely phenylglyoxylate carboxy-lyase behaved in a manner consistent with it being soluble.

Iodoacetate, iodoacetamide and NEM, which are generally thought of as thiol inhibitors, had little effect on either enzyme except at high concentrations (Table 13) probably reflecting reaction with parts

of the molecule other than with thiol groups (e.g. amino or carboxylate groups: Webb, 1966; Means & Feaney, 1971). The more reactive mercurials, p-chloromercuribenzoate and $HgCl_2$, were found to inhibit both enzymes, however. This implies that they both require a thiol group for activity. The greater inhibition of D-mandelate dehydrogenase by both reagents compared to the inhibition of L-mandelate dehydrogenase may suggest that its thiol group is more accessible. L-Mandelate dehydrogenase is inhibited to a greater extent by $HgCl_2$ than by p-chloromercuribenzoate confirming that some steric factors may impede the reaction. However, as there are various mechanisms by which such reagents can inhibit enzymes, interpretation of the inhibition by sulphydryl reagents should be exercised with care (see e.g. Webb, 1966). Similarly, in the absence of pure enzyme and considering the necessity for including BSA in the reaction mixture, it seemed premature to do more complicated, although obvious, experiments such as studies on the time course of inactivation and on the reversibility of inhibition.

Both D- and L-mandelate dehydrogenase also show similarity in their lack of inhibition by a number of metal-chelating reagents. No inhibition of either enzyme was seen with EDTA, o-phenanthroline, 8-hydroxyquinoline, 2,2-bipyridyl, KCN or NaN_3 at concentrations which are normally inhibitory for metal-requiring enzymes (Table 14). The inhibition or stimulation of either enzyme at very high concentrations of some of these reagents was probably due to non-specific effects. An accurate assessment of any cation requirement will

require purification of the enzymes. No attempt was made to see if addition of metal ions stimulated either D- or L-mandelate dehydrogenase activity.

Oxalate inhibited both enzymes almost identically (Table 14). Although oxalate can give enzyme inhibition by chelating calcium ions, this seems unlikely here in view of the lack of inhibition by the other chelating agents. Interestingly, Snoswell (1966), measuring the activity of membrane-bound D- and L-lactate dehydrogenases from Lactobacillus arabinosus by following the reduction of DCIP, found oxalate to be a potent competitive inhibitor of both enzymes. He found complete inhibition of both activities in crude extracts with 1mM oxalate. This inhibition seems likely to be due to oxalate acting as a substrate analogue. A similar mechanism could be envisaged for the inhibition of D- and L-mandelate dehydrogenases by oxalate.

Similarities in the temperature-dependence of enzyme activity (Fig. 15) and pH optimum values (Fig.16) for both enzymes were seen, although L-mandelate dehydrogenase seems less sensitive to pH, especially low values.

The mandelate dehydrogenases differ in their substrate specificities. Each enzyme is absolutely specific for the oxidation of one stereoisomer only (e.g. Table 8, 18 & 21). However, some similarity may exist between the active sites of both enzymes as it is possible to inhibit L-mandelate dehydrogenase with D-mandelate, and vice versa (Results

5.1). The inhibition of L-mandelate dehydrogenase is competitive, but the K_i is high (approx. 4mM) suggesting that D-mandelate binds weakly to L-mandelate dehydrogenase. Conversely the K_m for L-mandelate dehydrogenase is very low (about 200μM). Although low enzyme activities made it difficult to classify the type of inhibition of D-mandelate dehydrogenase by L-mandelate, it was clear that L-mandelate was a better inhibitor of D-mandelate dehydrogenase. The K_i for D-mandelate dehydrogenase (about 700μM) is more comparable to the K_m for the substrate of this enzyme (approx. 550μM), which suggests that although the dehydrogenation reaction is specific, the binding of substrate might be less so. Alternatively, there may be two binding sites on each enzyme, one site for L-mandelate and one for D-mandelate. Such sites would be associated with either catalysis or inhibition as appropriate.

The most striking difference between the two dehydrogenases was found in their heat stabilities. D-Mandelate dehydrogenase is heat-labile, whilst L-mandelate dehydrogenase is relatively heat-stable. This difference was shown in intact bacteria (Fig. 3) and in extracts (Results 1.2 & Fig. 20). Both the evolved and the original D-mandelate dehydrogenases in mutants 219 and 364 (derived ultimately from strains NCIB8250 and EBF65/65 respectively) showed similarities in their heat labilities (Fig. 20). Since heat lability in newly evolved enzymes is often postulated to be due to mutations which have occurred in the structural gene of the parent enzyme (see Hartley, 1974; Clarke, 1974) it was first supposed that the greater heat lability of the D-mandelate dehydrogenase was because it had arisen from some other enzyme by mutation. The similar heat lability of the original D-mandelate dehydrogenase found in strain EBF65/65, however, suggests

that this is not the correct explanation. Further both the original and evolved L-mandelate dehydrogenases in these mutants were alike in their relative heat stability. It may be interesting to compare the heat stability of L-mandelate dehydrogenase of P. putida A.3.12 (ATCC12633) and the D-mandelate dehydrogenase of P. putida ATCC17426 with those from the Acinetobacter strains. A difference in stability between enzymes using different stereoisomers as substrates does not seem to be unique. Snoswell (1963, 1966) found the NAD^+-independent D-lactate dehydrogenase of Lactobacillus arabinosus was less stable during both purification and storage compared with the L-lactate dehydrogenase from the same organism. In Propionobacterium pentosaceum the D-specific lactate dehydrogenase was found to be heat-labile (Molinari & Lara, 1960): the activity of the purified enzyme was completely destroyed by boiling for 3min, and only 15% of activity remained after heating to 47°C for 3min. In this organism there appears to be no L-lactate dehydrogenase activity.

Presumably the lability of D-mandelate dehydrogenase is due to complete or partial thermal denaturation of the enzyme. An alternative, but unlikely, explanation for lability could involve some form of proteolytic cleavage, the greater loss of enzyme activity as temperature increased would then be due to the proteolytic reaction rate. Since L-mandelate dehydrogenase seems unaffected, the protease involved would have to be very specific. The greater stability of the L-mandelate dehydrogenase may suggest the molecule is less susceptible to unfolding of the polypeptide chain.

The primary electron acceptor for either L- or D-mandelate dehydrogenase is not known. Both enzymes can transfer electrons to DCIP, although D-mandelate dehydrogenase seems less efficient in this respect

than L-mandelate dehydrogenase as the original assay procedure was unsuitable for measurement of D-mandelate dehydrogenase activity. It does nonetheless reduce DCIP directly if sufficient protein is used (Fig. 8 & Fig. 11 C to D). PMS can also accept electrons from both enzymes (iv, Table 12; Discussion 2.2). Kennedy (1967) showed that L-mandelate dehydrogenase activity was unaffected by either NAD^+ or $NADP^+$ and this was confirmed in the present work (p.103). Thus L-mandelate dehydrogenase seems to be an $NAD^\pm$-independent membrane-bound enzyme. Although FAD and FMN (p.103) have no effect on L-mandelate dehydrogenase activity, it is possible that a flavin could be involved if it were tightly bound to the enzyme. For example mammalian succinate dehydrogenase, a membrane-bound NAD^+-independent dehydrogenase, has a tightly bound FAD moiety which acts as the primary electron acceptor (see Hatefi & Stiggall, 1976). Alternatively, as the enzyme is membrane-bound, there may be some form of interaction between the enzyme and a component of the electron transport chain, perhaps a cytochrome. Mammalian cytochrome c cannot act as an electron acceptor (p.103). At the moment there seems to be some confusion as to which cytochromes are present in this organism. Whittaker (1974) reported that cytochromes a_1, a_2 and b were present, whilst Jones & King (1972) detected b, c and a_2.

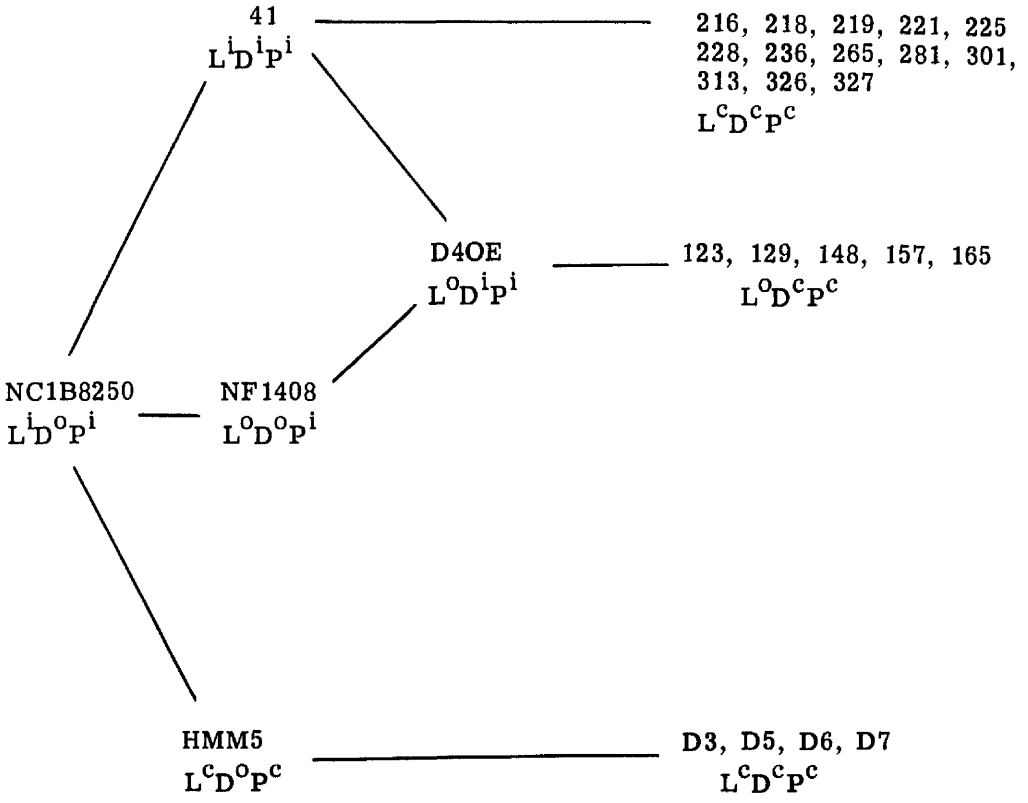
Nothing is known concerning the nature of the native electron acceptors of D-mandelate dehydrogenase. As the D- and L-mandelate dehydrogenases are so similar it will be interesting to see if they have the same electron acceptor. It is tempting to speculate that they may resemble the succinate or lactate dehydrogenases of other organisms which have been mentioned already, or the malate dehydrogenase

present in the same strain (NCIB8250; Jones & King, 1972). All these enzymes are membrane-bound, $NAD(P)^+$-independent and either flavin or possibly cytochrome-linked.

4. The regulation of the evolved mandelate dehydrogenases

When L-mandelate-utilizing strains evolve a D-mandelate dehydrogenase, there seems good evidence that this enzyme is regulated co-ordinately with L-mandelate dehydrogenase, i.e. as part of the R_1 regulon. Thus in mutant 41, the evolved enzyme is induced by R_1 regulon inducers, including the gratuitous inducer thiophenoxyacetate, and is both susceptible to catabolite repression by succinate and is repressed when the anti-inducer for the R_1 regulon enzymes, 2-phenyl-propionate, is present (Table 15; Fig. 17).

This conclusion was reinforced by an examination of the mutants isolated from strains 41, D4OE and HMM5 (see summary Scheme 14). When mutant strains constitutive for phenylglyoxylate carboxy-lyase synthesis were isolated from strain 41 they were also found to be constitutive for both D- and L-mandelate dehydrogenase activities (Table 17a). Similarly when this procedure was applied to strain D4OE (which has an inducible D-mandelate dehydrogenase, but lacks L-mandelate dehydrogenase activity), mutants constitutive for phenylglyoxylate carboxy-lyase and for D-mandelate dehydrogenase were obtained (Table 17b). These results would not be expected if the D-mandelate dehydrogenase was controlled in a manner unrelated to that of the R_1 regulon. Furthermore, when mutants able to grow on D-mandelate were isolated from strain HMM5, which has constitutive L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase activities, all were found to synthesize D-mandelate dehydrogenase constitutively as well (Table 17c) If D-mandelate



L L-mandelate dehydrogenase

D D-mandelate dehydrogenase

P phenylglyoxylate carboxy-lyase

o no enzyme activity

i inducible enzyme activity

c constitutive enzyme activity

A SUMMARY OF MUTANTS ISOLATED FROM Acinetobacter calcoaceticus NCIB8250 (see also Table 1)

Scheme 14

dehydrogenase was controlled separately, mutants with an inducible D-mandelate dehydrogenase might have been expected.

A similar pattern of regulation was found in mutants ultimately derived from wild-type strain EBF65/65. Mutants synthesizing phenylglyoxylate carboxy-lyase constitutively were isolated from strain C48 (the auxotroph of strain EBF65/65), which has an inducible phenylglyoxylate carboxy-lyase and D-mandelate dehydrogenase, and from strain 61c, a mutant of strain C48 which has evolved an inducible L-mandelate dehydrogenase (see Scheme 13). In both cases the mutants synthesized D-mandelate dehydrogenase constitutively and, when appropriate, L-mandelate dehydrogenase.

The co-ordinate control of D- and L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase in strain 41 was indicated by the correlation between the specific activities of these enzymes under a variety of conditions (Fig. 17). The lowest correlation coefficient, 0.90, was found between L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase, which have previously been shown to be co-ordinately expressed (Introduction p.19). Although the criteria of co-ordinate control are generally accepted (e.g. p.274 Lewin, 1974; Stanier & Ornston, 1973), quantitative tests of co-ordinacy, for instance by calculation of the correlation coefficient, are often not made. Instead graphs showing the relative rates of enzyme activity are presented with no statistical test of the results (e.g. Ames & Garry, 1959; Glansdorff & Sand, 1965; Ornston, 1966; Cánovas & Stanier, 1967; Cook & Cain, 1974). In order to see how the value of 0.90 compared with values in the literature, the graphs of Englesberg et al.

(1965) were traced and subjected to statistical analysis. A value of 0.89 was calculated for the correlation between the L-arabinose permease and L-arabinose isomerase in *araC*^{C} mutants of *E. coli*. These two enzymes are regarded as showing co-ordinate expression (Englesberg *et al.*, 1965). Furthermore, the data presented showing correlation between L-ribulokinase and L-arabinose isomerase, two enzymes actually translated from the same polycistronic mRNA (p.323 Lewin, 1974), gave a calculated correlation coefficient of 0.95. This is comparable to the correlation coefficients of 0.97 shown between D- and L-mandelate dehydrogenase specific activities and 0.94 between D-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase. Livingstone & Fewson (1972) calculated the correlation coefficient between the differential rates of synthesis of L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase to be 0.98 in wild-type strain NCIB8250. The differential rate of enzyme synthesis takes account of the activity of the new protein being synthesized rather than total protein, and so provides a much more accurate measure of enzyme activity during conditions of induction, repression and anti-induction. The calculation of differential rates of enzyme synthesis is time consuming and so specific activities are generally used in preference, as in this thesis and in many papers (e.g. Ames & Garry, 1959; Glansdorff & Sand, 1965; Ornston, 1966; Cánovas & Stanier, 1967).

Since expression of enzyme activity differed up to ten-fold amongst the various mutants (Table 17a, b & c), activities of the enzymes for each class of mutant derived ultimately from strain NCIB8250 were analysed statistically by the method used for mutant 41. Good correlation was found between D-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase for mutants isolated from strain D4OE

(correlation coefficient 0.94 from the data in Table 17b) and strain HMM5 (correlation coefficient 0.98 from the data in Table 17c). Furthermore D-mandelate dehydrogenase activity is co-ordinate with L-mandelate dehydrogenase in mutants derived from strain HMM5 (correlation coefficient 0.93 from the data in Table 17c).

The situation in mutants derived from strain 41 proved somewhat more complicated, with D- and L-mandelate dehydrogenase showing some co-ordinacy (correlation coefficient 0.74 from the data in Table 17a) but neither D- nor L-mandelate dehydrogenase exhibiting co-ordinacy with phenylglyoxylate carboxy-lyase (correlation coefficient -0.11 and -0.55 respectively from the data in Table 17a). Preliminary analysis of the data suggested that these mutants could be divided into two classes with respect to their growth rates on L-glutamate. For specific growth rates (μ) between 0.46 to 0.69h^{-1} (mutants 216, 225, 228, 236, 267 and 301), which were similar to the growth rates for mutants derived from strains D4OE and HMM5, fair correlation was found between D- and L-mandelate dehydrogenase (correlation coefficient 0.84) and between D- or L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase (correlation coefficient 0.98 and 0.83 respectively). For mutants which grew more slowly on L-glutamate (μ = 0.21 to 0.35h^{-1}; mutants 219, 221, 288 313 and 326), however, D- and L-mandelate dehydrogenase still show good co-ordinacy (correlation coefficient 0.94) but neither was co-ordinate with phenylglyoxylate carboxy-lyase (correlation coefficient -0.63 and -0.55 respectively). It does not seem as though mutant 327 can be included in either of these catagories since even though the growth rate was 0.49h^{-1}, the activity of phenylglyoxylate carboxy-lyase was high and so in this respect mutant 327 is similar to those mutants that grew slowly. There appears therefore to be a genuine difference in specific

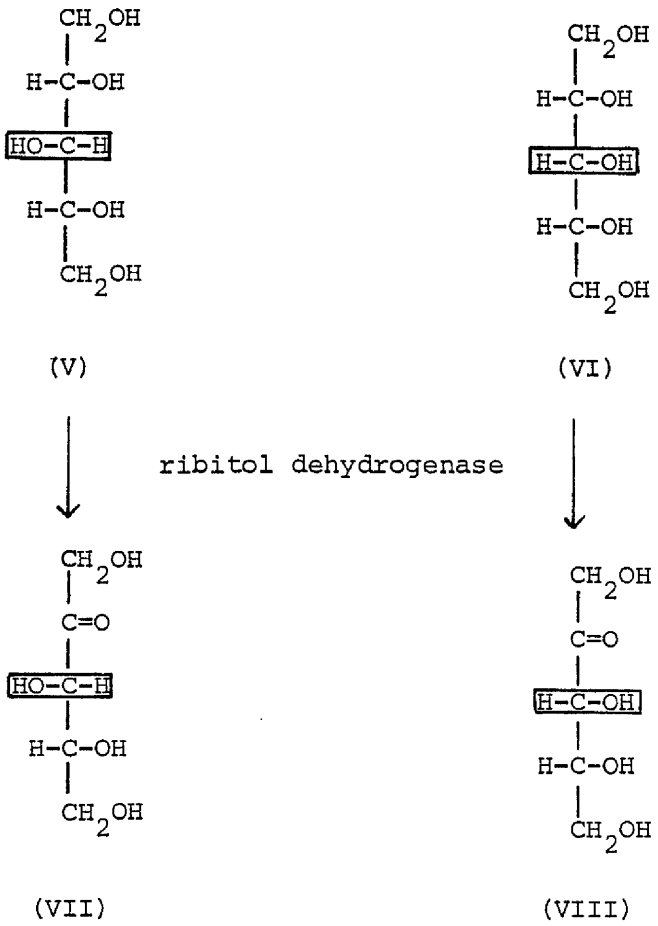
activities of the enzymes in these mutants derived from strain 41. However it is not possible to draw any firm conclusions regarding the nature of these differences. The specific activities of many enzymes are known to be affected by growth rate, for instance Dean (1972) listed examples of enzymes whose specific activity increased, decreased or went through maxima or minima as growth rate increased. It may be speculated that although the three R_1 enzymes are subject to the same overall control, differences may exist in some subordinate aspect of control so that growth rate may affect the expression of phenylglyoxylate carboyx-lyase differently from that of D- or L-mandelate dehydrogenase. This will be expanded in Section 6 of the Discussion.

5. Evolution of the new mandelate dehydrogenase

If, as this work suggests, the evolved enzyme is regulated in the same way as the original enzyme, a pertinent question is the mechanism by which the evolved activity arises.

As already discussed (Section 2.1 p.167) the evolution of a D-mandelate dehydrogenase in mutant 41, derived from strain NCIB8250, proceeds without loss of L-mandelate dehydrogenase activity. Similarly the evolution of L-mandelate dehydrogenase in mutant 61c (derived from strain C48) occurs without loss of D-mandelate dehydrogenase activity (Table 21). Consequently, if in the former case D-mandelate dehydrogenase arises as a result of mutation of L-mandelate dehydrogenase, then there must have been two (or more) copies of the original L-mandelate dehydrogenase gene with one copy undergoing mutation so that it produced an altered enzyme which could metabolize D-mandelate. If this were the case, we might expect twice as much L-mandelate dehydrogenase activity in wild-type strain NCIB8250 as compared with mutant 41. However, as shown in Table 18 this was not the case; instead both strains show similar activities and the ratio of L-mandelate dehydrogenase to phenylglyoxylate carboxy-lyase was similar for both organisms. It is difficult to completely discount this possibility, however, since expression of duplicate genes need not be proportional to the actual number of gene copies (see Introduction p.38).

The apparent lack of suitable mutation producing D-mandelate dehydrogenase from L-mandelate dehydrogenase is not totally unexpected since it is the asymmetric carbon atom of mandelate which is involved in the dehydrogenation reaction. Thus, different stereospecificities about this atom might require very different active sites in the appropriate enzymes. In fact, Hartley (1974) concluded that it was very difficult to evolve ribitol dehydrogenase to utilize xylitol efficiently by mutations in the structural gene because xylitol (V) differs from ribitol (VI) in having an altered stereospecificity of the hydroxyl group <u>adjacent</u> to the carbon atom involved in the dehydrogenation reaction. Thus the reaction products are D-xylulose (VII) and D-ribulose respectively (VIII):



Hartley's work may be contrasted with that of Clarke (1974) who found evolution of new substrate specificities in the amidase of P. aeruginosa to occur quite readily. Here, however, it is probable that the catalytic site remains unaltered since the reaction involved is essentially the same, i.e.

$$XCONHR + H_2O \longrightarrow XCOOH + NH_2R$$

(where R is usually H)

Mutations leading to amino acid changes in regions which determine the accommodation of the side chains of the amide substrate probably results during alteration of substrate specificity.

Another possibility for the origin of a new dehydrogenase is recruitment, and possibly mutation, of an enzyme from another metabolic pathway. This also seems unlikely in view of the similar regulation of evolved and original activity in mutants derived from strain NCIB8250 and EBF65/65.

A further mechanism by which the new dehydrogenase may have arisen is by expression of a previously 'silent' gene. The term 'silent' gene in this thesis refers to a genetic locus containing information for a potentially active polypeptide although this potential is not realized in the bacterium either because the information is not transcribed or is translated as an inactive product. A changed environment might then cause selection for mutants in which the 'silent' function becomes re-expressed.

A cycle of loss of expression and re-expression of the mandelate-metabolizing enzymes in A. calcoaceticus was observed by Beggs & Fewson (1977) with the following lineage of mutants:

$$HMM5 \longrightarrow J8 \longrightarrow J8/R1$$

They took strain HMM5, which is constitutive for the R_1 regulon enzymes,

and isolated mutant strains such as J8 which, although constitutively synthesizing large amounts of benzaldehyde dehydrogenase I, had no L-mandelate dehydrogenase or phenylglyoxylate carboxy-lyase activity, even under normal inducing conditions. Further selection of mutant J8 gave mutant J8/R1 which constitutively synthesized all the R_1 regulon enzymes again. Although the molecular mechanism underlying these results is not known, they do suggest that genetic loci capable of coding for functional polypeptides may be rendered unexpressible and then regain expression with relative ease.

It may be speculated that those strains possessing either D- or L-mandelate dehydrogenase activities are derived from ancestral strains possessing both enzymes, one of which has become 'silent'. In such a case selection for the absent activity might be expected to produce mutants in which the 'silent' gene is re-expressed. This would suggest that similarities should be found between an evolved enzyme activity and the corresponding original activity in another strain sharing a common ancestral origin. In this respect a few preliminary results (Table 22, Fig. 20) suggest that the evolved D-mandelate dehydrogenase in mutant 219, derived ultimately from strain NCIB8250, resembles the original enzyme present in mutant 364, derived ultimately from strain EBF65/65. Similarly the evolved L-mandelate dehydrogenase in mutant 364 resembles the original enzyme in mutant 219. These results suggest the existence of similar genes for both D- and L-mandelate dehydrogenase in both strain NCIB8250 and EBF65/65, differing only in whether they are expressed or not. Further comparison of D- and L-mandelate dehydrogenases from other Acinetobacter strains would be required to substantiate this proposal. It would also be interesting to compare the D- and L-mandelate dehydrogenases from Pseudomonas spp.

and fungi. Comparison of the enzymes in the various P. putida strains could be particularly interesting because some strains have evolved a D-mandelate dehydrogenase (e.g. strain ATCC17426), whilst others have an L-mandelate dehydrogenase and a racemase (e.g. strain ATCC12633; see p.10 of Introduction and Scheme 3).

The mechanism whereby the D- or L-mandelate genes are rendered 'silent' is unknown. At present, distinction between an untranscribed gene and one which is transcribed but wholly or partly translated as an inactive product is not possible. However when the mandelate dehydrogenases are purified it may be possible to test for the presence of inactive polypeptides using immunological techniques. This would involve raising antibodies against purified dehydrogenases for use as probes to detect the presence of any inactive polypeptides.

The possibility that 'silence' of mandelate dehydrogenases in different strains may result from different molecular mechanisms is suggested by comparison of the frequencies of occurrence of mutants possessing evolved activity (Table 20 & 24). It should be noted that estimates of frequencies of occurrence of mutants based upon growth in a few flasks are subject to potentially large errors. For example if the 'true' rate of occurrence of mutants would lead us to expect, say, three flasks to show growth, then there is about a 45% chance that less than three flasks will show growth, and about a 20% chance that less than two flasks will show growth (estimated from Poisson probability paper). Conversely, there is about a 35% chance of at least four flasks showing growth and about a 20% chance of at least five flasks showing growth. Thus one to five flasks may show growth, although the 'true' number should be three. Hence quite a large variation in estimated frequency of occurrence of mutants might be expected. To overcome this problem the number of flasks used in each experiment

would have to be increased, but as most of the experiments presented on Table 24 for instance involved up to forty flasks, each experiment would then become difficult in practical terms.

Another source of error exists in the estimates of frequency of occurrence of mutants in the absence of phenylglyoxylate in the enrichment medium. Here, the number of bacteria capable of mutation was calculated assuming that there were 10^8 bacteria ml^{-1} of nutrient broth culture (Ahlquist, 1974). This value may be an underestimate in these experiments and so the frequency of occurrence of mutants will be overestimated. When phenylglyoxylate was present in the medium, however, the number of bacteria capable of mutation was calculated by plating and counting colonies.

It should be also noted that in these experiments the 'frequency of mutation' has been calculated rather than the 'rate of mutation'. This latter parameter also accounts for the generation time and is probably the best estimate to use, but the most difficult to calculate.

Even with all these errors in mind, the frequency of occurrence of mutants from strains NCIB8250 and NF1408 able to utilize D-mandelate appears to be similar. In addition, with strain NF1408, the frequency of occurrence of revertants able to utilize L-mandelate and evolvants able to utilize D-mandelate were similar. On the other hand a considerably greater frequency of occurrence of mutants of strain C48 able to utilize L-mandelate was found (Table 20). This higher frequency of occurrence of mutants probably does not reflect a higher inherent rate of mutation in strains derived from EBF65/65 in comparison to strains derived from NCIB8250. Thus the frequency of occurrence of mutants able to synthesize phenylglyoxylate carboxy-lyase constitutively in mutants ultimately derived from strain NCIB8250 is about 10^{-7} (Fewson

et al., 1978; Table 16) and, although the actual rates were not calculated, approximately the same frequency of mutation was observed when mutants (354, 355, 364, 369, 381 and 386) were derived from strains C48 and 61c (i.e. ultimately from strain EBF65/65).

Therefore the different frequency of mutation to evolve a D-mandelate dehydrogenase in strain NCIB8250 and an L-mandelate dehydrogenase in strain EBF65/65 may reflect different mechanisms by which the genes were 'silenced' originally.

The important role played by permeation during evolution of novel metabolic capabilities has been previously noted (see Introduction p.29 & 32). Since strain NCIB8250 presents a permeability barrier to D, L-mandelate (Cook & Fewson, 1972b) the mechanism whereby a novel mandelate gains access to the bacterium is of some importance. Cook & Fewson (1972b) showed that induction of the R_1 regulon enzymes leads to an increase in permeability of D, L-mandelate in strain NCIB8250. It might now be instructive to repeat and extend this work using both stereoisomers with strains NCIB8250 and EBF65/65.

If an inducible permeation mechanism is involved, the experimental evolution of novel mandelate stereoisomer utilization might suggest a fairly non-specific transport system. An alternative explanation could involve concurrent 'silence' of mandelate dehydrogenase and a permeation component, both becoming re-expressed together. Lastly it is possible that no transport system is required in view of the membrane location of both dehydrogenases. In this case the dehydrogenase itself would act either as a 'permease' for the mandelate, or to directly transport the product of the dehydrogenation reaction, phenylglyoxylate, into the bacterium. Indeed, the suggestion that

mandelate oxidation and transport are linked was made by Cook & Fewson (1972b) to explain the low permeability of mandelate observed with mutant NF1408 which lacks L-mandelate dehydrogenase.

Finally, in view of the similarities between both mandelate dehydrogenases, it may be speculated that they are derived from the same ancestral protein. Possibly, evolution of this protein in different strains could have led to the existence of rudimentary L- and D-mandelate dehydrogenases which were brought together by genetic transfer. Alternatively, as the ancestral protein was probably only weakly active, increasing total enzyme by means of gene duplication would have been favoured. The gene copies could then have evolved separately to give L- or D-mandelate dehydrogenase. If antibodies are made specifically directed against either L- or D-mandelate dehydrogenase it may be possible to investigate this problem by looking for cross-reactivity. The ultimate test will be an examination of the amino acid sequence of the two enzymes.

With respect to present day organisms the work of Baumann et al. (1968) and Fewson (using strains isolated by Dr. E. Barnes from decayed chicken carcasses) shows that most strains of Acinetobacter which can utilize mandelate use both stereoisomers (see Introduction p. 9). The higher proportion of strains utilizing D- and L-mandelate in those selected by Dr E. Barnes may suggest a selective pressure for D- and L-mandelate utilization in decayed chicken carcass. In addition, work described in this thesis suggests that those strains which utilize only one isomer may have a 'silent' gene whose potential product can utilize the other isomer. If evolution of D- or L-mandelate dehydrogenase in separate strains had occurred, a higher proportion having activity of only one enzyme might have been expected. Thus, the

evolution of D- and L-mandelate dehydrogenase from duplicates of an ancestral protein may be the most likely possibility.

On the other hand most strains of Acinetobacter utilize neither mandelate nor phenylglyoxylate. Does this mean that these strains have become 'silent' for the entire R_1 regulon or has the mandelate pathway evolved in a few strains but not in the vast majority? If the pathway is merely 'silent' in the majority of strains then it may be possible experimentally to evolve activities of different mandelate pathway enzymes. A strain of possible significance with respect to these speculations is strain 8 of Baumann et al. (1968) which does not utilize either stereoisomer of mandelate but does utilize phenylglyoxylate. Thus it apparently has an intermediate position between the extremes of no R_1 regulon activity and complete R_1 regulon activity. It would be interesting to see if D- and/or L-mandelate dehydrogenase activity could be evolved in this strain. Furthermore this strain might prove useful in transformation studies as recipient for D- and L-mandelate dehydrogenase genes from both Acinetobacter and Pseudomonas strains.

6. Speculations concerning control and organization of the genes of the mandelate pathway

The enzymes of the R_1 regulon are induced by phenylglyoxylate (Livingstone & Fewson, 1972). It is tempting to speculate that a regulatory protein exists which controls the expression of the R_1 regulon enzymes, the activity of this protein being modulated by phenylglyoxylate. Such a protein could function by binding to a DNA site either to repress enzyme synthesis, becoming unbound in the presence of phenylglyoxylate (negative control), or normally being unbound to the DNA site but binding to activate synthesis in the presence of phenylglyoxylate (positive control). Mutation to constitutivity would be expected to be frequent in the former case, but rare in the latter (Hood et al., 1975), since most mutations in the regulatory protein would prevent its binding to the DNA site. As constitutive mutants arise with a reasonably high frequency of approximately 10^{-7} (Fewson et al., 1978; Table 16) it is possible that the R_1 regulon is negatively controlled.

The organization of the genes of the R_1 regulon is unknown. In mutants J8 (Beggs & Fewson, 1977) and CO4211 (Fewson et al., 1978) the regulation of benzaldehyde dehydrogenase I is no longer co-ordinate with L-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase. Furthermore, in some mutants derived from strain 41 which grow slowly, phenylglyoxylate carboxy-lyase is no longer co-ordinate with D- and L-mandelate dehydrogenase (see Section 4 of the Discussion). This suggests that while the mandelate enzymes are controlled as one regulon, different enzymes may be expressed independently because of a sub-ordinate system of control such as separate operators. Possibly in

these mutants derived from strain 41 the D- and L-mandelate dehydrogenase could be catabolite repressed to a greater extent than phenylglyoxylate carboxy-lyase.

The high frequency of co-transformation of D- and L-mandelate dehydrogenase activity using DNA from strain NF1408 as recipient (Ahlquist, 1974) suggests close linkage between these two enzymes. Towner (1978)* has located the approximate position of L-mandelate dehydrogenase on the apparently circular genome of strain EBF65/65 using RP4-mediated conjugation. It will now be interesting to locate the original D-mandelate dehydrogenase gene present in strain EBF65/65 with respect to the position of the evolved L-mandelate dehydrogenase and to the genes for the other R_1 enzymes. Rosenberg (1971), using generalised transduction, was able to comment on the linkage of the genes coding for phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase in P. aeruginosa. Although he suggested that the genes were closely linked, the evidence as to whether these genes are regulated as a single operon was ambiguous. Unfortunately no other work has been done on the genetic analysis of the mandelate pathway.

*Towner (1978) used mutant 61c isolated in the work described in this thesis to map the L-mandelate dehydrogenase gene, but wrongly attributed its isolation to Dr C.A. Fewson.

7. General Conclusions and Prospects

The evolved D- and L-mandelate dehydrogenases in mutants derived from strains NCIB8250 and EBF65/65 are now known to be controlled as part of the R_1 regulon.

The most reasonable suggestion seems to be that there is a gene coding for D- or L-mandelate dehydrogenase in strain NCIB8250 and EBF65/65 respectively but for some unknown reason the gene product is undetected either because it is not transcribed, or because its product is an inactive polypeptide.

More information about the enzymes themselves must await the development of solubilization and purification procedures. Ultimately comparison depending on antigenic properties, amino acid composition and sequence could be made. Comparison with other microbial and fungal mandelate dehydrogenases, and their regulation, may provide insights into the evolution of this pathway and of the microorganisms themselves.

Genetic manipulations may clarify the mechanisms whereby novel D- or L-mandelate dehydrogenases evolve and may throw light on the mechanisms whereby control of the R_1 regulon is achieved.

REFERENCES

Adelberg, E.A., Mandel, M. & Chen, G.C.C. (1965) Biochem. Biophys. Res. Commun. 18, 788-795

Ahlquist, E.F. (1974) M.Sc. Thesis, University of Glasgow

Ames, B.N. & Garry, B. (1959) Proc. Nat. Acad. Sci. U.S. 45, 1453-1461

Arraj, J.A. & Campbell, J.H. (1975) J. Bacteriol. 124, 849-856

Axelrod, J. & Saavedra, J.M. (1977) Nature (London) 265, 501-504

Bartha, R. & Atlas, R.M. (1977) Adv. Appl. Microbiol. 22, 226-266

Baumann, P. (1968) J. Bacteriol. 96, 39-42

Baumann, P., Doudoroff, M. & Stanier, R.Y. (1968) J. Bacteriol. 95, 1520-1541

Beggs, J.D. (1974) Ph.D. Thesis, University of Glasgow

Beggs, J.D., Cook, A.M. & Fewson, C.A. (1976) J. Gen. Microbiol. 96, 365-374

Beggs, J.D. & Fewson, C.A. (1977) J. Gen. Microbiol. 103, 127-140

Betz, J.L., Brown, P.R., Smyth, M.J. & Clarke, P.H. (1974) Nature (London) 247, 261-264

Bhat, S.G., Ramanarayanan, M. & Vaidyanathan, C.S. (1973) Biochem. Biophys. Res. Commun. 52, 834-842

Bhat, S.G. & Vaidyanathan, C.S. (1976a) Arch. Biochem. Biophys.176, 314-323

Bhat, S.G. & Vaidyanathan, C.S. (1976b) Eur. J. Biochem. 68, 323-331

Brammar, W.J., Clarke,P.H. & Skinner, A.J. (1967) J. Gen. Microbiol. 47, 87-102

Brown, J.E. & Clarke, P.H. (1970) J. Gen. Microbiol. 64, 329-342

Brown, P.R. & Clarke, P.H. (1972) J. Gen. Microbiol. 70, 287-298

Cain, R.B., Bilton, R.F. & Darrah, J.A. (1968) Biochem. J. 108, 797-828

Campbell, J.H., Lengyel, J.A. & Langridge, J. (1973) Proc. Nat. Acad. Sci. U.S. 70, 1841-1845

Camyre, K.P. & Mortlock, R.P. (1965) J. Bacteriol. 90, 1157-1158

Cánovas, J.L. & Stanier, R.Y. (1967) Eur. J. Biochem. 1, 289-300

Cánovas, J.L., Ornston, L.N. & Stanier, R.Y. (1967) Science 156, 1695-1699

Carles, J. & Bourguet, M. (1957) Comp. Rend. Soc. Biol. 151, 383-387

Chen, R.F. (1967) J. Biol. Chem. 242, 173-181

Clarke, P.H. (1974) in Evolution in the Microbial World, Symp. Soc. Gen. Microbiol. 24 (Carlile, M.J. & Skehel, J.J., eds.) 183-217, C.U.P. London

Cook, A.M. & Fewson, C.A. (1972a) Biochem. J. 127,77P

Cook, A.M. & Fewson, C.A. (1972b) Biochim. Biophys. Acta 290, 384-388

Cook, A.M. & Fewson, C.A. (1973) Biochim. Biophys. Acta 320, 214-216

Cook, A.M., Beggs, J.D. & Fewson, C.A. (1975) J. Gen. Microbiol. 91, 325-337

Cook, K.A. & Cain, R.B. (1974) J. Gen. Microbiol. 85, 37-50

Cowan, S.T. & Steel, K.J. (1965) Manual for the Identification of Medical Bacteria, C.U.P., London

Dagley, S. (1971) Adv. Microbiol. Physiol. 6, 1-46

Dean, A.C.R. (1972) J. Appl. Chem. Biotechnol. 22, 245-259 reprinted in Environmental Control of Cell Synthesis and Function (Dean, A.C.R., Pirt, S.J. & Tempest, D.W. eds.) 245-259, Academic Press, London & New York

Dickie, C.W. & Regnier, J.O. (1978) J. Amer. Vet. Med. Ass. 172, 357-359

El Marsi, A.M., Smith, N.J. & Williams, R.T. (1958) Biochem. J. 68, 199-204

Englesberg, E., Irr, J., Power, J. & Lee, N. (1965) J. Bacteriol. 90, 946-957

Fee, J.A., Hegeman, G.D. & Kenyon, G.L. (1974a) Biochemistry 13, 2528-2532

Fee, J.A., Hegeman, G.D. & Kenyon, G.L. (1974b) Biochemistry 13, 2533-2538

Fewson, C.A. (1967a) J. Gen. Microbiol. 46, 255-266

Fewson, C.A. (1967b) J. Gen. Microbiol. 48, 107-110

Fewson, C.A. & Foote, A. (1976) Proc. Soc. Gen. Microbiol. 3, 177

Fewson, C.A., Livingstone, A. & Roach, P.J. (1970) Biochem. J. 118, 53P

Fewson, C.A., Beggs, J.D., Seenan, E. & Ahlquist, E.F. (1976) Biochem. Soc. Trans. 4, 614-615

Fewson, C.A., Livingstone, A. & Moyes, H.M. (1978) J. Gen. Microbiol. 106, 233-239

Fewster, M.E. & Hall, D.A. (1951) Nature (London) 168, 78-79

Foster, J.F. (1960) in the Plasma Proteins (Putman, F.W. ed.) 1, 179-233, Academic Press, London & New York

Glansdorff, N. & Sand, G. (1965) Biochim. Biophys. Acta 108, 308-311

Glew, R.H., Moellering, R.C. & Kunz, L.J. (1977) Medicine 56, 79-97

Guerola, N., Ingraham, J.L. & Cerdá-Olmedo, E. (1971) Nature (London) 230, 122-125

Gunsalus, C.F., Stanier, R.Y. & Gunsalus, I.C. (1953a) J. Bacteriol. 66, 548-553

Gunsalus, I.C., Gunsalus, C.F. & Stanier, R.Y. (1953b) J. Bacteriol. 66, 538-542

Gunter, S.E. (1953) J. Bacteriol. 66, 341-346

Gutnick, D.L. & Rosenberg, E. (1977) Ann. Rev. Microbiol. 31, 379-396

Haddock, A.M. (1973) Honours Dissertation, University of Glasgow

Hall, B.G. & Hartl, D.L. (1974) Genetics 76, 391-400

Hartley, B.S. (1974) in Evolution in the Microbial World, Symp. Soc. Gen. Microbiol. 24 (Carlile, M.J. & Skehel, J.J. eds.) 151-182, C.U.P., London

Harvey, N.L., Fewson, C.A. & Holms, W.H. (1968) Lab. Pract. 17, 1134-1136

Hatefi, Y. (1978) in Methods in Enzymology (Fleischer, S. & Packer, L. eds.) 53, 27-35, Academic Press, London & New York

Hegeman, G.D. (1966a) J. Bacteriol. 91, 1140-1154

Hegeman, G.D. (1966b) J. Bacteriol. 91, 1155-1160

Hegeman, G.D. (1966c) J. Bacteriol. 91, 1161-1167

Hegeman, G.D. & Kenyon, G.L. (1970) Biochemistry 9, 4036-4042

Hegeman, G.D. & Rosenberg, S.L. (1970) Ann. Rev. Microbiol. 24, 429-462

Hegeman, G.D., Rosenberg, S.L. & Kenyon, G.L. (1970) Biochemistry 9, 4029-4035

Henrichsen, J. & Blom, J. (1975) Acta. Pathol. Microbiol. Scand. Sect. B 83, 103-115

Henriksen, S.D. (1973) Bacteriol. Rev. 37, 522-561

Henriksen, S.D. (1976) Ann. Rev. Microbiol. 30, 63-83

Herman, N.J. & Juni, E. (1974) J. Virol. 13, 46-52

Higgins, S.J. & Mandelstam, J. (1972a) Biochem. J. 126, 901-916

Higgins, S.J. & Mandelstam, J. (1972b) Biochem. J. 126, 917-922

Holms, W.H. & Bennett, P.M. (1971) J. Gen. Microbiol. 65, 57-68

Hood, L.E., Wilson, J.H. & Wood, W.B. (1975) Molecular Biology of Eucaryotic Cells p.6, W.A. Benjamin Inc., California

Horowitz, N.H. (1945) Proc. Nat. Acad. Sci. U.S. 31, 153-157

Horowitz, N.H. (1965) in Evolving Genes and Proteins (Bryson, V. & Vogel, H.J., eds.) 15-24, Academic Press, London & New York

Inderleid, C.B. & Mortlock, R.P. (1977) J. Mol. Evol. 9, 181-190

Ito, H., Sato, T. & Iizuka, H. (1976) Agr. Biol. Chem. 40, 867-873

Jamaluddin, M., Subba Rao, P.V. & Vaidyanathan, C.S. (1970) J. Bacteriol. 101, 786-793

Jensen, R.A. (1976) Ann. Rev. Microbiol. 30, 409-425

Jones, M. & King, H.K. (1972) FEBS Lett. 22, 277-279

Juni, E. (1978) Ann. Rev. Microbiol. 32, 349-371

Kennedy, S.I.T. (1967) M.Sc. Thesis, University of Glasgow

Kennedy, S.I.T. & Fewson, C.A. (1968a) J. Gen. Microbiol. 53, 259-273

Kennedy, S.I.T. & Fewson, C.A. (1968b) Biochem. J. 107, 497-506

Kishmore, G. Sugumaran, M. & Vaidyanathan, C.S. (1974) Biochim. Biophys. Res. Commun. 56, 851-859

Koch, A.L. (1972) Genetics 72, 297-316

Lancaster, J.D. (1971) Honours Dissertation, University of Glasgow

Leblanc, D.G. & Mortlock, R.P. (1971) J. Bacteriol. 106, 90-96

Lee, J.S. & Harrison, J.M. (1968) Appl. Microbiol. 16, 1937-1938

Lerner, S.A., Wu, T.T., Lin, E.C.C. (1964) Science 146, 1313-1315

Lewin, B. (1974) Gene Expression Vol. I, John Wiley & Sons, London

Lewis, E.B. (1951) Cold Spring Harbor Symp. Quant. Biol. 16, 159-174

Livingstone, A. (1970) Ph.D. Thesis, University of Glasgow

Livingstone, A. & Fewson, C.A. (1972) Biochem. J. 130, 937-946

Livingstone, A.L., Fewson, C.A., Kennedy, S.I.T. & Zatman, L.J. (1972) Biochem. J. 130, 927-935

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

Maas, W.K. & Clark, A.J. (1964) J. Mol. Biol. 8, 365-370

Maggio, E.T., Kenyon, G.L., Mildvan, A.S. & Hegeman, G.D. (1975) Biochemistry 14, 1131-1139

Mandelstam, J. & Jacoby, G.A. (1965) Biochem. J. 94, 569-577

Matson, I.C. (1974) Honours Dissertation, University of Glasgow

Means, G.E. & Feeney, R.E. (1971) Chemical Modification of Proteins pp.105-114, Holden Day, California

Merck Index (1976) Windholz, M. (ed.) Ninth Edition, p.81, Merck & Co., USA

Mislow, K. (1951) J. Amer. Chem. Soc. 73, 3954-3956

Molinari, R. & Lara, F.J.S. (1960) Biochem. J. 75, 57-65

Mortlock, R.P. Fossit, D.D., Petering, D.H. & Wood, W.A. (1965) Proc. Nat. Acad. Sci. U.S. 54, 572-579

Moyes, H.M. (1976) Honours Dissertation, University of Glasgow

Moyes, H.M. & Fewson, C.A. (1976) Biochem. Soc. Trans. 4, 1105-1106

Nakada, D. & Nozu, K. (1958) Japan. J. Microbiol. 2, 137-141

Oliver, E.J. & Mortlock, R.P. (1971a) J. Bacteriol. 108, 287-292

Oliver, E.J. & Mortlock, R.P. (1971b) J. Bacteriol. 108, 293-299

Ornston, L.N. (1966) J. Biol. Chem. 241, 3800-3810

Ornston, L.N. (1971) Bacteriol. Rev. 35, 87-116

Ornston, L.N. & Parke, D. (1977) Current Topics in Cellular Regulation 12, 209-262

Ornston, L.N. & Stanier, R.Y. (1966) J. Biol. Chem. 241, 3776-3786

Otsuji, H. & Ikeda, M. (1970) Brit. J. Industr. Med. 27, 150-154

Pardee, A.B., Benz, E.J., St. Peter, D.A., Krieger, J.A., Meuth, M. & Trieshmann, H.W. (1971) J. Biol. Chem. 246, 6792-6796

Patel, R.N., Meagher, R.B. & Ornston, L.N. (1973) Biochemistry 12, 3531-3537

Peters, T. & Reed. R.G. (1978) in Albumin : Structure, Biosynthesis, Function (Peters, T. & Sjöholm, I., eds.) 11-20, Pergamon Press, London

Propenoe, E.A., Aronson, R.B. & Slyke, D.D.V. (1969) Arch. Biochem. Biophys. 133, 286-292

Rajasekhar, M., Muniyappa, L. & Keshara Murthy, B.S. (1978) Vet. Rec. 102, 557

Ramakrishna Rao, D.N. & Vaidyanathan, C.S. (1977) Can. J. Microbiol. 23, 1496-1499

Ramanarayanan, M. & Vaidyanathan, C.S. (1973) Indian J. Biochem. Biophys. 10, 254-256

Reddy, C.C. & Vaidyanathan, C.S. (1975) Biochim.Biophys. Acta 384, 46-57

Reiner, A.M. (1971) J. Bacteriol. 108, 89-94

Reiner, A.M. (1972) J. Biol. Chem. 247, 4960-4965

Reiner, A.M. & Hegeman, G.D. (1971) Biochemistry 10, 2530-2536

Rhoads, R.E., Hutton, J.J. & Udenfriend, S. (1967) Arch. Biochem. Biophys. 122, 805-807

Rigby, P.W.J., Burleigh, B.D. & Hartley, B.S. (1974) Nature (London) 251, 200-204

Riley, M. & Anilionis, A. (1978) Ann. Rev. Microbiol. 32, 519-560

Robinson, J. & Cooper, J.M. (1970) Anal. Biochem. 33, 390-399

Rosenberg, S.L. (1971) J. Bacteriol. 108, 1257-1269

Rosenthal, S.L. (1978) in Glucose Nonfermenting Gram-Negative Bacteria in Clinical Microbiology (Gilardi, G.L., ed.) 105-117, CRC Press Inc., Florida

Rosett, T. (1965) Appl. Microbiol. 13, 254-256

Smyth, P.F. & Clarke, P.H. (1975) J. Gen. Microbiol. 90, 91-99

Snoswell, A.M. (1963) Biochim. Biophys. Acta 77, 7-19

Snoswell, A.M. (1966) in Methods in Enzymology (Wood, W.A., ed.) 9, 321-327, Academic Press, London & New York

Sridhara, S., Wu, T.T., Chused, T.M. & Lin, E.C.C. (1969) J. Bacteriol. 98, 87-95

Stanier, R.Y. (1947a) J. Bacteriol. 54, 339-348

Stanier, R.Y. (1947b) J. Bacteriol. 55, 477-494

Stanier, R.Y. & Ornston, N.L. (1973) in Adv. Microbial Physiol. (Rose, A.H. & Tempest, D.W., eds.) 9, 81-151, Academic Press, London & New York

Stanier, R.Y., Gunsalus, I.C. & Gunsalus, C.F. (1953) J. Bacteriol. 66, 543-547

Stanier, R.Y., Palleroni, N.J. & Doudoroff, M. (1966) J. Gen. Microbiol. 43, 159-271

Stevens, F.J. & Wu, T.T. (1976) J. Gen. Microbiol. 97, 257-265

Stevenson, I.L. & Mandelstam, J. (1965) Biochem. J. 96, 354-362

Sugumaran, M., Ramanarayanan, M. & Vaidyanathan, C.S. (1973) FEBS Lett. 29, 62-77

Suhara, K., Takemori, S. & Katagari, M. (1969) Arch. Biochem. Biophys. 130, 422-429

Taylor, R.P. (1977) in Albumin Structure, Function and Uses (Rosenoer, V.M., Oratz, M. & Rothschild, M.A., eds.) 183-201, Pergamon Press, London

Thornley, M.J., Ingram, M. & Barnes, E.M. (1960) J. Appl. Bact. 23, 487-498

Tiwari, N.P. & Maxcy, R.B. (1972) J. Food Science 37, 901-903

Towner, K.J. (1978) J. Gen. Microbiol. 104, 175-180

Towner, K.J. & Vivian, A. (1976) J. Gen. Microbiol. 93, 355-360

Towner, K.J. & Vivian, A. (1977) J. Gen. Microbiol. 101, 167-171

Véron, M. (1966) Ann. Inst. Pasteur 111, 671-709

von Graevenitz, A. (1977) Ann. Rev. Microbiol. 31, 447-471

Weast, R.C. (1977) Handbook of Chemistry & Physics, 59th Edition
1978-1979, C-93, CRC Press, USA

Webb, J.L. (1966) Enzyme and Metabolic Inhibitors, Vol. II, pp.635-653
Academic Press, London & New York

Weil-Malherbe, H. (1966) Biochem. J. 101, 169-175

Whittaker, P.A. (1974) Microbios 4, 65-70

Williams, P.A. & Worsey, M.J. (1976) Biochem. Soc. Trans. 4, 466-468

Wu, T.T. (1976a) Biochim. Biophys. Acta 428, 656-663

Wu, T.T. (1976b) J. Gen. Microbiol. 94, 246-256

Wu, T.T. (1978) CRC Crit. Rev. Microbiol. 6, 33-51

Wu. T.T., Lin, E.C. & Tanaka, S. (1968) J. Bacteriol. 96, 447-456

Yeh, W.K., Davis, G., Fletcher, P. & Ornston, L.N. (1978) J. Biol. Chem.
253, 4920-4923